University of Alberta

T cell development and the diversification and selection of the *Xenopus* T cell receptor beta chain repertoire

by

Erika L. Meier ©

A thesis submitted to the faculty of graduate studies and research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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December 4th, 2002.

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled, T cell development and the diversification and selection of the *Xenopus* T cell receptor beta chain repertoire, by Erika L. Meier in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in (Molecular Biology and Genetics).

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Kenneth L. Roy

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Kathy Magor

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December 4th, 2002.

To Allan for his patience, faith and support.

To my parents for teaching me everything I really needed to know.

To Tracy as partial payment of a substantial debt.

Abstract

Vertebrates protect themselves from infection by somatically generating unique receptors termed immunoglobulins (Ig) or T cell receptors (TCR) from building blocks scattered along a chromosome. The combinatorial joining of variable (V), diversity (D), joining (J) and constant regions create a gene coding for a unique receptor and provides the receptor diversity needed to recognize rapidly evolving pathogens. T cell receptors are heterodimers composed of either an α and a β chain or a γ and a δ chain. This thesis addresses the early development of T cells in the anuran frog *Xenopus* and the onset of this receptor building rearrangement for the TCR β chain.

The segments which rearrange to form the receptor were exhaustively cloned in hybrids of *X. laevis* and *X. gilli*. The *laevis* locus contains 25 Vβ segments, 24 of which are expressed. The *gilli* locus contains at least 25 segments, 20 of which rearrange and are transcribed. 17 and 19 Vβ families are used in *laevis* and *gilli*, respectively. The *laevis* and *gilli* loci contain 19 and 13 Jβ segments, respectively. An antiserum to a co- receptor molecule, the CD3ε chain of the TCR, is shown to stain *Xenopus* T cells and allowed the visualization

of T cell subset in a cold-blooded vertebrate. In ontogeny, CD3ε positive cells are first observed in the stomach on day 5 after fertilization, one day before full TCR β rearrangements are found in the thymus and gut/liver. All Vβ segments are used by tadpoles and adults. TCR diversity seems most limited by the low number of T cells in tadpoles.

The repertoire selected during development has been studied in two clonal lines derived from siblings, LG3 and LG15 that share the TCR β locus but differ at their major histocompatibility complex (MHC) and other genes. The usage of some Vβ segments was correlated to developmental changes in class I MHC expression, and in some cases to MHC selection on different backgrounds of the animals. In a theoretical model, it is proposed that the number of active T cells and thus the TCR β repertoire in the body is further constrained by changes in ambient temperature.

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The pure and simple truth is rarely pure and simple.

-Oscar Wilde

"How do you know that? Are you an expert on labyrinths?
'No, I am merely citing an ancient text I once read."
"And by observing this rule you get out?
"Almost never, as far as I know. But we will try it just the same".
-Umberto Eco, The Name of the Rose

Symbols, Nomenclature and Abbreviations

α: alpha
β: beta
γ: gamma
δ: delta
ε: epsilon
ζ: zeta
ad: fill to
Amp: ampicillin
APBS: amphibian PBS
AP: alkaline phosphatase
APC: antigen presenting cell
AS: antisense
BCR: B cell receptor
BSA: bovine serum albumin
β_2m: beta 2 microglobulin
C: constant region
CDR: complementary determining region
ConA: concanavalin A
CTL: cytotoxicT lymphocyte
Cyt: cytoplasmic tail
D: diversity region
DAPI: 4,6-diamidino-2-phenylindole
DC: dendritic cell
DEPC: diethyl pyrocarbonate
dH_2O: distilled water
DIG: digoxygenin
DMF: dimethylformamide
DTT: dithiothreitol
dpf: days post-fertilization
dsDNA: double-stranded DNA
EDTA: ethylenediaminetetraacetic acid
EtBr: ethidium bromide
FCS: fetal calf serum
FITC : fluorescein isothiocyanate
FR: framework region
fsDNA: fish sperm DNA
H4: hypervariable region
hpf: hours post-fertilization

Igsf: immunoglobulin superfamily
IPTG: Isopropyl-β-D-thio-galactopyranoside
ITAM: immunoreceptor tyrosine-based activation motif
IP: immunoprecipitation
J: joining region
LG: one of a series of lines of *X. laevis/gilli* hybrids
LPS: lipopolysaccharide
mAb: monoclonal antibody
MHC: major histocompatibility complex
MLR: mixed lymphocyte reaction
Mr: molecular weight
Mya: millions of years ago
NK: natural killer cell
NR: non-reducing
NUP: nested universal primer
OD: optical density
pAb: polyclonal antibody
pCD3ε: polyclonal antiserum to CD3ε
PBL: peripheral blood lymphocytes
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PHA: phytohaemagglutinin
pMHC: Peptide MHC complexes
Prot.K: proteinase K
R: reducing
RAG: recombination activating gene
RT: reverse transcriptase
Rxn: reaction
S: sense
SDS: Sodium dodecyl sulphate
SDS-PAGE: polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate
SSC: standard saline citrate
SsDNA: salmon sperm DNA
TAP: transporter associated with antigen processing
TEA: triethanolamine
TCR: T cell receptor
TH: thyroid hormone
TM: transmembrane region
TR: Texas red fluorochrome

V: variable region
UPM: universal primer mix
UTR: untranslated region
X-Gal: 5-bromo-4-chloro-3-indolyl-β -D-galactoside

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Chapter 1

Introduction

1.0 Introduction

All multicellular organisms studied to date have evolved mechanisms to protect themselves from invasion by parasites or infectious microorganisms [1]. A central challenge all immune systems face is the need to distinguish self from non-self so immune mechanisms do not begin to attack self/host tissues [2]. Furthermore, a context for immune recognition may be needed so that dangerous non-self (potential pathogens) can be quickly and effectively neutralized while ignoring innocuous non-self (components in dust and food etc.). Comparisons of the immune responses of many species have attempted to form a picture of how immunity evolved and whether phylogenetic relationships can be found in the approaches organisms use for immunity. This thesis will focus on issues of diversity in the immune response.

Immune responses are traditionally grouped into two categories: innate and adaptive. Innate immunity, is ancient and found in all studied metazoans whereas adaptive immunity has only been found in jawed vertebrates and is present in addition to the older innate system [3]. One of the major distinctions between innate and adaptive immunity is that the innate system relies on genomically encoded receptors for the recognition of very conserved components of potential pathogens. In contrast, the vertebrate adaptive immune response can, through DNA recombination, somatically generate an immense diversity of receptors which can recognize almost any component (antigen) (reviewed in [4-6]). By comparing adaptive immunity in several species at key phylogenetic positions, we hope to better understand how variability is generated and maintained in this system.

1.1 Innate Immunity: germline encoded diversity

The term innate immunity is currently used to describe immune mechanisms that are not encompassed by the vertebrate adaptive response.

Innate immunity consists of both non-specific responses to damage or foreign particles, and specific recognition of different classes of potential pathogens. Many organisms can form physical barriers between healthy and infected or damaged tissues (reviewed in [1]). Foreign particles can also be coated (opsonized), allowing them to be lysed or recognized and taken up by phagocytic cells ([7] and as reviewed in [1]). Specific cell types which phagocytose foreign particles are also common [reviewed in [1, 7].

Specific recognition of very conserved components of potential pathogens is mediated by receptors encoded in the host genome. The need for a discrimination of self from non-self also requires that these receptors recognize only those conserved components that are found only in the microbe but not in the host organism [2]. Conserved microbial components, dubbed PAMPs (pathogen associated molecular patterns), such as the mannans found in fungal cell walls or the double stranded RNA which is a part of some viral life cycles, are vital for the survival of the microbe and are likely to remain invariant over long evolutionary time spans [8]. There will also be little variation between related microbes, allowing the recognition of one microbial component to alert the organism to many groups of potential pathogens. If there is any overlap between the components recognized by the immune system and components found in the self, it could lead to destruction of self-tissue. Organisms as diverse as plants, insects and vertebrates use the same ancient signaling cascade after recognizing conserved components of viral and bacterial nucleic acids and cell walls [9, 10]. The receptors involved in recognition do not always have the same function nor do they necessarily lead to the same functional outcomes. For instance, in *Drosophila,* Toll receptors are used to receive chemical signals from other cells whereas in mice similar receptors are used to directly recognize non-self structures [9, 11-13]. In *C. elegans* the system is present but does not participate in immunity [14]. Therefore the same signaling system is conserved but the sensor and the output differs [15, 16].

In addition to responses against microbial pathogens, organisms have used specific immunity to maintain their individual integrity and to better compete for resources with other members of their own species. This can be seen in when two sponges or corals make contact. A barrier forms between sponges which recognize each other as non-self [17-19]. In some sponge species, specialized cell types are also present which can destroy non-self cells at this junction [20, 21]. In corals, recognition of allopolymorphism leads to the programmed cell death (apoptosis) of the tissues in the zone of contact between incompatible hosts [22].

Innate responses, unlike adaptive responses, generally do not become more robust or faster with multiple exposures of an individual to the pathogen. New receptors for invariant components can only be developed in the species on an evolutionary timescale.

Because microbes have generation times orders of magnitude faster than most metazoans, the potential for evolution to diversify a pathogen component or to evolve a mechanism to fool or block the very conserved and slow evolving innate response will always be faster than the production of counter-responses from the host [23]. Despite this, the majority of multicellular life is dependent on innate immunity for survival [1]. This is easier to understand in light of recent studies showing a considerable diversity of immune receptors in invertebrates. In molluscs, molecules called fibrinogen related proteins (FREPs) are involved in resistance to parasites. FREPs are incredibly polymorphic in the population and each snail possesses 40 or more different FREP genes [24]. In the sea urchin, the scavenger receptor system contains over 1200 genes [24] and a new class of very diverse LPS inducible molecules has recently been reported [24].

Diversity in these innate receptors is probably necessary and selected. However, one can imagine that species with long life-spans may not be able to trust even a large array of innate responses, for over their lifespans they are more likely to come into contact with a pathogen which can interfere with genomically encoded pattern recognition. Clearly, developing an adaptive system based on somatic generation of large numbers of different recognition structures would provide an evolutionary advantage. This assumption is supported by the presence of an ancient form of immunity, present in fungi, plants and animals referred to as RNA interference (RNAi). This system recognizes the double stranded RNA characteristic of some viruses and retrotransposons, and cuts it into small 21-25 base pair (bp) pieces. These pieces are then used as a specific "receptor" allowing nuclease to specifically destroy transcripts with homologous sequences, and protect the organism from the same pathogen [25]. RNAi shows many of the characteristics used to define adaptive immunity including extreme specificity and, in some cases, long lasting protection (memory) [26, 27]. This system however is only useful in providing specificity for pathogens with a double stranded phase in their lifecycle. A mechanism where small pieces of protein could be recognized as self or non-self would be an advantage to the organism, allowing it to monitor protein production in the same way as RNAi monitors transcripts in the cell. Such a system evolved in jawed vertebrates.

1.2 Adaptive immunity: somatically acquired diversity

Adaptive immunity first developed in jawed vertebrates (gnathostomes) (as reviewed in [1]). This new form of immunity was characterized by the production of a wide, almost unlimited repertoire of unique receptors by somatic DNA rearrangement in each individual of the species. These receptors could recognize not only conserved but also unique components of foreign substances. Thus, specificity for a vast array of antigens is generated before those antigens are encountered. This new adaptive immune response also becomes faster and most robust upon repeated exposure to the same component (antigen). This specificity and long lasting immunological memory allowed immune responses to be tailored to the survival of the individual in addition to the species.

The main difference between innate and adaptive immunity is the question of diversity. The specificity in innate immunity is reliant on a limited number of genomically encoded receptors which recognize conserved pathogens and evolve their specificities over millions of years, thus benefiting the species. In contrast, adaptive immunity is based upon the production of very diverse receptors (Immunoglobulins; Ig and T cell receptors; TCR) specific even for components with which the organism has not come into contact. This diversity is produced somatically, i.e. during the lifetime of the individual. The genes encoding these somatically acquired receptors do not exist physically as single expressible units but are assembled by DNA rearrangement from segments scattered along a chromosome. We inherit our immune receptor genes as a sort of do it yourself kit which each individual uses to generate a diverse set of unique receptors. It has therefore been stated that "the adaptive response retains the immune memory of the individual, but the innate response retains the evolutionary memory of the species" [7]. In both innate and adaptive immunity the need for diversity in the population is apparent but DNA rearrangement allows jawed vertebrates to generate millions of different specificities rather than hundreds or thousands that could be reasonably expected to be encoded in some innate systems.

Although receptor diversity is hallmark of the adaptive immune system, it is still not clear how much diversity is actually needed to protect an organism, especially when one considers that invertebrates thrive without adaptive immunity based upon rearranging receptors. Also, because most of our knowledge about adaptive immunity comes from a study of placental mammals, we know little about how requirements for diversity may vary with the size, lifespan or lifecycle of an organism.

By comparing the adaptive immunity in many vertebrates we may approach this question because the vertebrate adaptive immune system first evolved in a poikilothermic vertebrate, it makes sense to study adaptive immunity in poikilothermic vertebrates. A better understanding of the selection pressures which have molded the system may help us understand why adaptive immunity is only found in jawed vertebrates and how it is maintained.

The work in this thesis describes the diversity present in the β chain of T cell receptors (TCR) in the anuran frog, *Xenopus*. In *Xenopus*, we can address several levels of TCR diversity in tadpoles and adult frogs. The diversity of the segments which rearrange to form the gene encoding the TCRβ chain is studied. As well, tadpoles are likely to possess one of the smallest T cell repertoires amongst gnathostomes. The ontogeny and diversity of T cells is studied in tadpoles. Frogs also are poikilothermic and undergo metamorphosis. In one organism it is possible to study diversity in the immune system of a vertebrate which differs so markedly from mammals.

1.2.1 General principles of the vertebrate adaptive immune system

The major cell types involved in adaptive immune responses are B and T lymphocytes and antigen presenting cells (APC).

1.2.1.1 B Lymphocytes

B lymphocytes, or B cells, are named after the Bursa of Fabricius in chicken where they were first described [28]. During its development, each B cell acquires (see section 1.2.1.2) a unique surface receptor which determines the specificity of that cell [29] [30]. This surface receptor is called an immunoglobulin. When B cells become activated they proliferate and a subset differentiates into effector cells which secrete a soluble form of the immunoglobulin (antibody) into the body fluids. The production of soluble immunoglobulins (antibodies) is referred to as a humoral response. A subset of the cells produced in the initial activation induced proliferation become long lived cells termed memory B cells. If the organism is exposed to the same antigen again, it is the memory cells that mediate a faster and more substantial secondary response [5].

1.2.1.2 T Lymphocytes

T cells, named for the thymus, the organ in which they mature, are involved in both humoral (antibody) and cellular responses [4]. During their development, T cells also acquire a unique surface receptor; the T cell receptor (TCR) (figure 1.1) [31]. The T cell receptor (TCR), a member of the immunoglobulin superfamily, determines the specificity of the T cell [32]. Heterodimers are composed of two disulfide linked chains, each composed of two immunoglobulin domains, a hinge region, a transmembrane domain and a short cytoplasmic tail. Only one type of heterodimer is expressed on an individual T cell.

The two main types of T cells, $\alpha\beta$ and $\gamma\delta$, form part of the vertebrate adaptive immune response and have been found in all jawed vertebrates studied to date [1].

1.2.1.3 The Major Histocompatibility Complex (MHC)

Both T cell receptors and immunoglobulins are members of the immunoglobulin superfamily and are thought to have descended from a common ancestral gene (as reviewed in [1]). However, immunoglobulins recognize antigens directly whereas TCRs recognize antigens which are presented bound to self molecules called class I and class II which are encoded by the major histocompatibility complex (MHC) [33, 34]. The cells which present antigens bound to MHC molecules are called antigen presenting cells or APCs. While the number of MHC loci expressed in an individual is quite low, class I and class II MHC molecules are extremely polymorphic within the population and are of two main types: class I and class II. The structure of class I and II MHC molecules is shown in figure 1.2.

1.2.1.3.1 MHC Class I

Class I MHC molecules are heterodimers consisting of a three domain heavy chain complexed with beta 2 microglobulin (β_2m). The binding cleft of classical class I molecules (class Ia) binds to short peptides derived from intracellular peptides (figure 1.3). Proteins are processed by intracellular machinery into short peptides; after being transported into the lumen of the Endoplasmic Recticulum (ER), they bind to the peptide binding groove of the MHC molecules and are transported to the cell surface where the peptide-MHC complex interacts with T cells. The T cell receptor interacts physically with both the self-MHC molecules and the presented peptide. Peptide MHC complexes will be referred to as, peptide-MHC (as reviewed in [35]). Class I MHC is expressed to varying extents on all nucleated cells [36]. It is therefore not found on mammalian erythrocytes but is readily found on the nucleated erythrocytes of non-mammalian species [36].

1.2.1.3.2 MHC Class II

Class II molecules are composed of an α and a β chain. This heterodimer forms a peptide binding groove very similar to class I. Class II molecules present peptides derived from extracellular proteins which are processed in the endosomal compartment of the cell. MHC class II molecules are expressed on a much smaller subset of cells, generally on so called antigen presenting cells or (APCs) such as activated dendritic cells and B cells. (as reviewed in [37]).

Because genes encoded in the MHC are physically close together, the set of MHC alleles contributed by one parent tends to be inherited as a set block and is referred to as a haplotype. Class I and class II MHC alleles are expressed co-dominantly. Therefore, in a heterozygous individual class II α chains from one haplotype can pair with class II β chains from the other haplotype (as reviewed in [35]). This is thought to present many different peptides to T cells improving the chance of an individual being able to permit the presentation of a peptide from any particular pathogen.

1.2.1.4 Clonal selection: evolution on a somatic timescale

Cells possessing T or B cell receptors (TCRs and BCRs), which recognize pathogens or altered self-cells in certain situations (immunological contexts) become activated and proliferate (clonal expansion). The expansion of these clones with receptors which best recognize invaders (clonal selection) results in extremely specific immune responses and the production of specific long lived memory cells which respond more quickly to future encounters with the same a pathogen component (antigen)[6, 38]. Each TCR and BCR has a unique specificity. The diversity of immune receptors available is therefore considerably more than the total number of genes in an individual. How is this diversity generated?

1.2.1.5 Somatic DNA recombination produces unique receptors

In each individual B and T cell, a gene encoding a unique receptor with a unique specificity is pieced together from an array of genomic TCR segments via a process of somatic DNA rearrangement (figure 1.4) [29-31]. This enables a relatively small amount of DNA to encode for a vast number of different receptors, each with a different specificity (see section 1.2.1.6). The genome contains arrays of distinct segments encoding variable (V), diversity (D), joining (J) and constant (C) region segments. Although all cells possess these genomic arrays of Ig and TCR segments, VDJ recombination occurs only in B, T and some subsets of natural killer (NK) cells termed NK T cells (reviewed by [39]). In T cells only the α, β, γ, and δ loci undergo full rearrangement. The IgH & IgL only rearrange in B cells. During recombination, two Recombination Activating Genes (RAG1 and RAG2) are expressed and their products form a recombinase very similar to that of some retro-transposons [40, 41]. The recombinase recognizes short sequences called recombination signal sequences (RSSs) flank the V, D, and J segments. The RSSs impart a directionality (orientation) to each segment facilitating the rearrangement of a D to a J segment in TCR γ and β and Ig heavy chains followed by a second rearrangement of a V segment to the DJ. In α, δ, and Ig light chains, only VJ rearrangement takes place, as there is no D segment.

1.2.1.6 Recombinational diversity in αβ T Cell receptors

Receptor diversity is generated through the combinatorial joining of V, D and J segments (β chain) and V and J segments (α chain). Further diversification can occur through the junctional flexibility at the points of joining (coding junctions). To join segments, the recombinase cuts the germline DNA and then rejoins these cut ends to generate a rearranged TCR gene. The enzyme cut sites are not precise so the point of rejoining can differ by several nucleotides on each coding end. Since the join is in the coding sequence this leads to a variation in the amino acids encoded at the junction. In adults, a "non-templated" DNA polymerase called terminal deoxyribonucleotidyl transferase (TdT) can add nucleotides to the cut ends before rejoining [42-45]. These "N nucleotides" further increase junctional diversity. Nucleotides can also be removed from the cut ends by endonucleases. Junctional flexibility, and nucleotide addition to and loss from the coding ends, can also produce stop codons or out of frame rearrangements (non-productive rearrangements).

There is a distinct order to somatic DNA rearrangement at the TCR loci. In vertebrates studied thus far, the production of γδ T cells precedes αβ T cells during thymic ontogeny ([46]reviewed in [4]). In the ontogeny of individual T cells, the TCR γ locus rearranges first. If this rearrangement is productive then the δ locus is rearranged. If no productive TCR γ rearrangement is produced on either the maternal or paternal γ locus, then the TCR β locus begins to rearrange, followed by the α locus ([47] and as reviewed in [48]). If a productive TCR γ rearrangement is produced, rearrangement of the second γ locus is inhibited and the δ locus undergoes rearrangement. If the rearrangement is unproductive, only then can the second γ locus undergo rearrangement. Similarly, the TCRβ locus undergoes rearrangement before the α locus. An unproductive rearrangement on one locus leads to VDJ recombination at the second locus. If this too is unproductive, the cell will not express a receptor and will die. TCR β chains are tested for complementarity with the TCR α chain by pairing with a pre-T α chain. Following a productive rearrangement at one β locus and the pairing of this chain with the pre-T α chain, rearrangement at the other β locus is stopped in a signaling dependent process. The pre-T cell undergoes a 1000 fold proliferation. In each of these cells, both TCR α loci undergo rearrangement simultaneously [49, 50]. Rearrangement at the β and γ loci are not fully independent however, as in mature $\gamma\delta$ T cells, one can find sterile Dβ-Jβ-Cβ and sterile Cβ transcripts (as reviewed in [48]). Sterile transcripts are not translated into proteins, but are important to note because many studies rely on measurements of transcription such as RT PCR products to determine the presence or absence of entire subsets of cells.

1.2.1.7 Allelic exclusion

Generally, T cells only express one functionally rearranged γ or β chain. This control is called allelic exclusion [51]. Once a productive γ or β chain is produced, rearrangement at the other locus is suppressed. However, this process is not perfect and 1% of mature peripheral $\alpha\beta$ T cells contain two productively rearranged β chains [52, 53]. Allelic exclusion at the α locus is not very stringent and this results in the expression of two productively rearranged α chains in 30% of peripheral human $\alpha\beta$ T cells and a small percentage of peripheral T cells in mice [54, 55]. T cells with two receptors have been hypothesized to be a potential autoimmunity risk because a T cell activated by interactions with one receptor may be able to attack self tissues through the second receptor [4]. It has recently been shown that T cells expressing two receptors are generally out-competed by normal T cells or become anergic (unresponsive) in the periphery and therefore probably of little consequence in normal immune responses. Other groups think these cells may be involved in regulation of immune responses or even extend the repertoire [50, 56].

1.2.2 T cell receptors

T cell receptors are of two types, $\alpha\beta$ and $\gamma\delta$. Here we will concentrate on the $\alpha\beta$ receptor.

Each chain of the $\alpha\beta$ heterodimer consists of two extracellular immunoglobulin domains and is linked to a short "hinge" peptide, a transmembrane domain, and a short cytoplasmic tail (see figure 1.5).

1.2.2.1 The variable domain

The first domain, a immunoglobulin superfamily (Igsf) V type domain, comes from a genetic region containing many variable segments. Each V segment encodes two highly conserved cysteines 60 to 75 aa apart, which, in the protein come together to form an intrachain disulfide bond [1, 4].

Genomically encoded variation in variable domain segments falls into three main regions termed complementarity determining regions: CDR1 and 2 and the hypervariable H4 region. CDRs 1 and 2 of both the α and β chains generally make contact with MHC molecules but have also been observed to make contact with the peptide contained in the MHC groove (reviewed in [57]). The H4 region is thought to interact with superantigens (see section 2.4.2). Superantigens are molecules produced by some pathogens which can bind the outside surfaces of both the TCR β chain and the MHC class II molecules thus linking the two together [58]. This causes a non-antigen specific proliferation of T cells possessing receptors which utilize specific variable segments and the subsequent death of those cells (as reviewed in [59]). A fourth hypervariable region, the CDR3 is encoded by the V-J-C or V-D-J-C join in α and β cells respectively and as such is the most variable of the four hypervariable regions [4].

The crystal structures for number of TCR/peptide/MHC complexes have been solved [57, 60-69]. The TCR heterodimer is positioned diagonally over the peptide/MHC complex. This brings/places the CDR1 and 2 loops of the TCR α and β chains over the amino and carboxy ends of the peptide respectively. The much more variable CDR3 loops of the TCR heterodimer generally make contact with the central portion of the peptide ([62] and as reviewed in [70]) (figure 1.6). CDR3 generally makes direct contact with the peptide in the MHC groove and it has been shown that mutations in this region can change the specificity of the receptor [71, 72]. Current dogma states that CDRs 1 and 2 of both chains make contact with residues in the α helices which form the walls of the MHC binding cleft, leaving the CDR3 loops to contact the peptide. This however, is a generalization, a recent review of the available crystallography data suggests that the TCR/peptide/MHC interaction is more complex than first thought, in that all three CDRs, as well as the H4 region can interact with the MHC, but do not always contact the same MHC residues [57].

1.2.2.2 TCR β constant regions

The second domain (see [4]) is a C1 type Igsf domain and is encoded by a genomic constant region segment. The connecting peptide contains a cysteine which forms a disulfide bond between the α and β chains. A lysine is present in all known TCRβ transmembrane regions and interacts with negative charges present in the transmembrane domains of the CD3 complex (see 1.2.2.3 below). The short cytoplasmic tails contain no signaling domains and are poorly conserved between species.

1.2.2.3 The CD3 complex

The T cell receptor heterodimer provides the antigen specificity of the cell but cannot be expressed on the cell surface or signal without an association with the CD3 complex (extensively reviewed in [4, 73, 74]). The CD3 complex is composed of three non-polymorphic/ invariant chains ε, γ/δ, ζ (figure 1.7). Mice and humans possess distinct γ and δ chains which are very similar and are encoded only 1.4 kb from each other [75]. Because of this similarity and proximity and the observation that in chickens [76], frogs [77,78], the Iberian ribbed newt (*Pleurodeles waltl*) (acc# AF397406) and chondrichthyan fish [79] there is only one CD3 γ/δ gene, a duplication is thought to have occurred after the divergence of mammals and birds [76]. This is supported by molecular data suggesting the divergence between the two chains occurred ~250 million years ago (mya) [80]. CD3ε, γ/δ, γ and δ all contain an extracellular immunoglobulin superfamily domain, a transmembrane domain with negatively charged residues and cytoplasmic tails containing immunoreceptor tyrosine-based activation motifs (ITAMs) (figure 1.7) [77, 81]. These ITAMs participate in signal transduction. In mammals, CD3ε forms heterodimers with CD3 γ and CD3 δ (as reviewed in [74]). In the other vertebrates studied to date, CD3ε is associated with γ/δ [78]. The negatively charged residues in the transmembrane domains of ε, γ/δ, γ and δ are thought to interact with positively charged residues in the

TCR α and β transmembrane domains and facilitate receptor assembly [82]; and as reviewed in [74]).

1.2.3 Types of T cells

1.2.3.1 γδ T cells

In mammalian and avian development, γδ T cells are the first functional T cells to enter the body periphery preceding αβ T cells. γδ T cells can be classified into two distinct groups. The first is a group of very conserved γδ TCRs utilizing the same canonical segments, which contain little or no junctional diversity. During ontogeny several waves of these T cells enter the periphery in a distinct order: the skin, reproductive tract and tongue, the lymph node and the intestine. The second group consists of γδ T cells with a very diverse usage of TCR segments and high junctional diversity. These cells are found in the intestine and the lungs. The function of γδ T cells remains somewhat unclear but is likely to differ between the two groups (reviewed in [83]). γδ T cells predominate during early human and mouse development, but are superceded by αβ T cells in adult life. In other animals such as sheep, cattle, rabbits, and chickens, γδ T-cells comprise up to 60% of the adult T cell population. [84, 85, 86, 262]. The significance of this is unknown. γδ T cells do not need antigen to be presented in the context of an MHC molecule but can directly recognize antigens in a manner more like antibodies e.g. Many of these antigens are not peptides but lipids or sugar moieties [87, 88]. Some γδ T cells however, are known to directly interact with non-classical MHC molecules such as T10 and T22 and can recognize bacterial glycolipids bound to the non-classical CD1 molecule [89-91].

1.2.3.2 αβ T cells

T cells which recognize an antigen in a specific context, can either act to kill that cell directly (cytotoxic T cell) or can direct immune responses either by direct cell to cell contact or through soluble mediators/messengers (helper T cell) [4].

1.2.3.2.1 Cytotoxic T cells

Cytotoxic T lymphocytes (CTLs) have TCRs, which interact with class I MHC-peptide complexes on other self-cells [4]. Because class I MHC presents peptides derived from intracellular proteins, these T cells can recognize cells which are virally infected or abnormal. When these T cells become activated, they can directly kill the infected or potentially cancerous cells, and are therefore referred to cytotoxic T cells. Cytotoxic T cells are characterized by their expression of the CD8 surface co-receptor (see below).

1.2.3.2.2 Helper T cells

Helper T cells interact with class II MHC/peptide complexes on the surface of APCs. These T cells cannot directly kill cells but can release chemical factors (cytokines and/or chemokines) which can direct the movement and reactivity of other cells in the body. Helper T cells are necessary for B cell activation leading to antibody production and for antibody class switching [4, 5]. Helper T cells are characterized by their expression of the CD4 surface co-receptor (see below).

1.2.4 The coreceptors: CD4 and CD8

CD4 and CD8 are referred to as co-receptors due to the important role they play in cell-to-cell adhesion and in enhancing T cell signaling [92, 93]. Thymocytes start out as $CD4^{+}CD8^{+}$ double positive (DP) cells. During selection in the thymus they become either $CD4^{+}$ or $CD8^{+}$ single positive (SP) before entering the periphery as mature T cells (as reviewed in [94]).

T cells that express CD4 are class II restricted and act as helper T cells whereas those with CD8 on their surface are class I restricted and serve as cytotoxic T lymphocytes (CTL). CD4 and CD8 are members of the immunoglobulin superfamily (Igsf).

CD4 is expressed as a monomer in association with the T cell receptor complex. It makes direct contact with class II molecules on the surface of the APC [95, 96].

CD8 is generally expressed as $\alpha\beta$ heterodimer. This heterodimer directly contacts the $\alpha3$ domain of MHC class I molecules on the surface of the APC. Some atypical populations of T cells such as intraepithelial lymphocytes (IELs), express CD8 $\alpha\alpha$ homodimers on their surfaces (see chapter 3).

1.2.5 Selection

1.2.5.1 Genomic vs. somatic selection

Until this point we have focused on the diversity of T and B cell receptors which somatic DNA rearrangement provides. However, like most good things, this diversity comes at a cost. Whereas innate receptors are selected at the genomic level for their ability to recognize conserved microbial components (antigens), the multitude of receptors which are randomly generated by somatic DNA recombination cannot be intrinsically specialized to recognize only antigens that are associated with pathogens. $\gamma\delta$ T cells with canonical CDR3 junctions, which are produced during fetal life, may be an exception to this generalization [84, 97-101]. Therefore, the distinction between self and non-self can no longer be achieved at the genomic level. This necessitates a selection process for testing such receptors for self-reactivity and eliminating or shutting off potentially dangerous cells (reviewed in [4, 35]). Experiments from the early 1900's showed that the immune system could mount responses to an amazing variety of substances and tissues. However, animals did not make immune responses to their own tissues. Paul Ehrlich, one of the founders of the science of immunology, suggested that the organism possessed potentially self reactive cells. This could lead to what he termed: "*horror autotoxicus*", an attack on self cells if they were not somehow made tolerant to self tissues (reviewed in [102]).

Much of modern immunology concerns itself with mechanisms involved in this tolerance to self, although many questions remain. It is clear however that tolerance to self is imposed at two levels: central tolerance and peripheral tolerance.

1.2.5.2 Central tolerance: positive and negative selection

In vertebrates, the thymus is the primary organ for T cell development (as reviewed in [1]). During early development the thymus segregates into two main zones; a cortex which surrounds an interior medulla. Precursor lymphocytes enter the outer cortex. As they proliferate, thier progeny move inwards towards the medulla, thymocytes begin to express large amounts of RAG and transcription begins at the TCR loci followed by the production of a unique TCR. Because the rearrangement of genomic segments is quite random, some receptors that are potentially self-reactive are produced. These self reactive T cells must be eliminated from the population before the remaining T cells enter the periphery. It is in the cortex that T cell receptors first encounter MHC molecules carrying peptides derived from self-proteins. As T cells move through the thymus, they are tested for their ability to recognize self peptide-MHC complexes. Two educational processes: positive and negative selection, help minimize the risk of releasing highly self-reactive T cells into the bloodstream and tissues of the body (periphery). This primary testing in the thymus is referred to as central tolerance induction.

1.2.5.2.1 Positive selection

T cells must recognize the specific alleles of MHC molecules present on the cells of the body. Such T cells are said to be self-MHC restricted [33, 103, 104]. This process of positive selection is not well defined but it is commonly thought that T cells that cannot recognize self MHC with an affinity above a certain threshold are not given a survival signal and die of "neglect"][105, 106]. T cells that express the CD8 co-receptor are class I MHC restricted and become cytotoxic T lymphocytes (CTLs). Cells with the CD4 co-receptor are restricted on class II MHC and act as helper T cells.

1.2.5.2.2 Negative selection

Self-MHC restricted T cells undergo the process of negative selection. Again, although the focus of intense research the mechanism of this process remains elusive. The current model hypothesizes T cells should not recognize self peptides bound to self MHC with too high an affinity, for such cells could lead to the destruction of self tissue. In the thymus, T cells are allowed to bind self peptide-self MHC complexes, and cells which bind with too high an affinity (or avidity) are destroyed (deleted) by an active process of apoptosis and not released into the rest of the body (periphery). Cells entering the periphery are therefore said to be self-MHC restricted and self-tolerant. The exact mechanisms of selection remain unclear. It is not even clear if the positive and negative selection occur sequentially or concurrently or whether both processes can take place in the cortex and medulla. What is clear is that ~98% of thymocytes die before leaving the thymus [107]. The few percent which are allowed into the periphery of the body contain functional T cell receptors and have been generally purged of "overly reactive" TCRs.

The above summary is a very general summary of selection. A more in depth discussion of current models of T cell selection can be found in section 6.4. Central tolerance plays an undeniably important role in protecting the individual from autoimmunity, however it does not, in practice, remove all self-reactive cells [35, 108, 109]. The process of negative selection is not so stringent as to eliminate all potentially self-reactive, cells possibly because this would decrease the size of the useable repertoire of T cells available in the periphery of the animal. A smaller number of peripheral cells would require the immune system to be more cross reactive to provide equivalent immunity which again might lead to cross-reactions with self antigens (extensively discussed in [110], and [35]). It is also true that not all self-antigens are available in the thymus due to access limitations or because they are only expressed at a time in the life cycle after central tolerance induction. The immune system must also ignore many outside non-pathogenic antigens to be efficient, therefore there must be a system for peripheral tolerance (reviewed in [102]).

1.2.5.3 Peripheral tolerance

Self-reactive T cells do enter the periphery occasionally. In mammals, the dangers of such events are minimized by the need for a second signal in the form of interaction with a co-stimulatory molecule for activation (as reviewed in [111]). For activation, naïve T cells require signaling through the TCR as well as a second signal through a co-stimulatory molecule which binds its ligand on an antigen presenting cell (APC). Co-stimulatory molecules are only found on specialized cells of the immune system, so called "professional antigen presenting cells" (APCs) which take up, process, and present both intracellular and extracellular antigens. This increases the likelihood of interactions with foreign MHC peptide complexes. T cells which recognize self without co-stimulation go into a state of non responsiveness, or anergy, where they cannot be stimulated. This process is thought to lead to peripheral tolerance. Additionally, the survival of naïve T cells in the periphery is dependent on continuing interactions with the selecting MHC [70, 112, 113]. Thus, T cells which cannot interact with MHC molecules die and are replaced by functional T cells.

1.2.6 Levels of diversity in the TCR repertoire

There are at least four different levels at which TCR repertoire can be studied, and the distinction between them is vital to our understanding of how diversity is generated and maintained in the immune system.

First, is the genomic repertoire, which refers to the diversity maintained in the genomic array of TCR segments. Second, is the diversity generated by somatic DNA rearrangement and junctional flexibility which gives the pre-selected repertoire. Third, is the selected repertoire, which is the fraction of the pre-selected repertoire which is selected on a particular MHC background. Fourth, is the peripheral repertoire, which is a measure of how much of the selected repertoire can be expressed in the periphery at any particular timepoint.

The peripheral repertoire is the repertoire which is available to the organism for protection. This is most likely the repertoire which is under the maximum evolutionary pressure. Smaller vertebrates have less immune cells in the periphery than larger vertebrates. In fact, there can be seven orders of magnitude difference between a tadpole and a large mammal (Louis Du Pasquier, personal communication). This should limit the diversity that can be expressed in the peripheral repertoire.

1.3 Comparative immunology

Although most of our knowledge of adaptive immunity is taken from mammals, the studies in other vertebrates provide relevant insights into the question of immunity. Indeed, adaptive immunity first appeared in a poikilothermic vertebrate and approximately two thirds of the 47 000 extant species of jawed vertebrates (gnathostomes) are poikilothermic (sometimes refered to as cold-blooded) [114].

All jawed vertebrates (gnathostomes) studied to date, including the model used in this thesis, the anuran amphibian *Xenopus laevis* , possess an adaptive immune system. The adaptive immune system is defined by rearranging receptors (TCR/Ig), antigen processing and presentation machinery (MHCIa, Ib, MHC II, LMP2, LMP7, tapasin etc), and genes involved in V-D-J rearrangement (RAG1, RAG2, TdT) (reviewed in [1]) (see figure 1.8).

There is no evidence for such a system in invertebrates or in the jawless vertebrates (agnathans) such as hagfish and lamprey (reviewed in [1]). Thus, the adaptive immune system seems to have developed in its entirety in the short evolutionary timespan between the divergence of jawed and jawless vertebrates [1, 115]. Agnathans lack the primary (thymus) or secondary (spleen) lymphoid organs present in other vertebrates [116]. Poikilothermic vertebrates differ from homeotherms in that germinal centers and lymph nodes are not present (figure 1.8). Still, as in homeotherms, the thymus is responsible for education of T cells and the spleen and the gut associated lymphoid tissues (GALT) are secondary peripheral lymphoid organs.

1.3.1 A general comparison TCR αβ diversity in vertebrates

The cloning and characterization of TCR segments from many different vertebrates will lead to a better understanding of how much genomically encoded variability is generally maintained in vertebrates. However, these studies are far from complete. Exhaustive cloning of segments or genomic sequencing of TCR loci has not been carried out in any poikilothermic vertebrate, leaving an incomplete picture of TCR diversity. Partial information is available for many species, and gives an initial view of the possible complexities involved.

1.3.1.1 Mice and humans

The murine and human genomic TCR β loci are well characterized [117-121]. Each consists of an array of Vβ segments located upstream, of two sets of D, J and C segments, each made up of one Dβ, 6-7 J β and a Cβ segment. Humans and mice possess 57 and 25 functional Vβ segments repectively [122-124]. When one combines the possible genomic diversity of both TCR chains with the juctional diversity added during recombination, it is estimated that a mouse can produce ~10^5 different αβ T cell receptors (as summarized by [125]). In humans this diversity is estimated at 1×10^{15} different receptors [126].

1.3.1.2 Birds

1.3.1.2.1 Chicken

The chicken possesses a very limited genomic repertoire of TCRβ segments in comparison with other species. There are only two Vβ families with 4 or 5 members each. Segments from both families can use the single Dβ segment, all 4 Jβ segments and the single constant region [127]. However Vβ1 and Vβ2 segments are used in functionally distinct T cell subsets. As well, Vβ1 is used before Vβ2 during T cell ontogeny in the embryo [127].

1.3.1.3 Reptiles

TCR genes have not been cloned in reptiles. It is predicted that reptiles possess a TCR because of their phylogenetic placement between amphibians and birds, two groups in which TCR has been found. They also have thymus and T cell function (graft rejection, MLR, etc. [128-130]).

1.3.1.4 Amphibians

1.3.1.4.1 The Mexican axolotl

TCR β chain rearrangements have been well characterized in the urodele amphibian axolotl. Over 250 rearrangements were sequenced showing axolotl possess at least 4 Cβ segments, 13 Vβ, 4 Dβ and 13 Jβ segments [131-136]. The genomic structure of the locus has been very difficult to study due to the immense size of the axolotl genome [131]. This precludes "normal Southern hybridizations" and makes screening genomic libraries very difficult [132]. Thus, it is unclear if the locus shares a multicluster or translocon architecture. Axolotl seem almost unique in their expansion of Cβ usage. In sharks, trout and in *Xenopus* there seems to be a reduction in the number of expressed loci. They have suggested that Cβ4 is so divergent from other Cβ segments that the ability to associate with the single Cα isotype found in axolotl is questioned [132]. Thus, it is clear that the combinatorial diversity of segments available to the axolotl has the capacity to far exceed that of mice and humans. More expression data will be needed to show the actual diversity of expression.

Four of the six Vα regions that can associate with Cα (Vα2, Vα3, Vα5 and Vα6) can also associate with Cδ, but no specific Vδ regions were found. This suggests that the axolotl TCR δ locus is nested within the TCR α locus, as in mammals, and that this organization has been present in all tetrapod vertebrates and must have been present in the common ancestor of Lissamphibians and mammals [137].

Axolotl are, ironically, known for their poor T cell responses. It remains possible that this diversity is somehow detrimental to the αβ T cell response, which might help account for the reduction in usage of Cβ regions in trout, sharks and *Xenopus*.

The anuran amphibian *Xenopus* is described below in section 1.3.3.1.1.

1.3.1.5 Fish

TCR segments have been cloned from cod, channel catfish, zebrafish, pufferfish and trout. Again, many of these studies have only reported a few segments, but suggest that there should be many more.

1.3.1.5.1 Cod

In cod only 4 TCRβ clones were characterized yielding 4 Vβ segments [138]. At least 3 Cβ segments were found and initial Southern hybridization experiments suggest that the locus is arranged in a translocon organization.

1.3.1.5.2 Channel catfish

7 Vβ segments have been cloned from the channel catfish along with 2 Jβ and 2 Cβ segments [139]. D segments were not described in the paper. Exhaustive cloning of TCR segments from channel catfish would be an extremely useful step towards an understanding of T cell function in poikilothermic vertebrates because immortalized T cell lines are available in catfish. 8 Vα and 4 Jα segments were also found associated with a single Cα segment [139].

1.3.1.5.3 Trout

Trout is by far the most completely studied model for TCR α and β diversity amongst poikilothermic vertebrates. 6 Vα and 32 Jα segments are found associated with a single Cα segment [140]. There may be more TCR α segments for only 40 clones were analyzed. To date 10 Vβ, 10 Jβ and one Dβ segment are found associated to a single Cβ segment. A genomic D J C cluster has been sequenced in trout which contains a single Dβ segment upstream of 10 Jβ segments and a single Cβ [141, 142]. Although only a single TCRβ locus has been reported to be active in trout, initial Southern hybridizations suggest that there may be other Cβ segments in the genome.

1.3.1.6 Cartilaginous fish

1.3.1.6.1 The horned shark

42 clones were characterized yielding 7 Vβ families, a single Dβ segment, 18 Jβ segments and at least 4 Cβ domains [143]. Pulsed field electrophoresis of genomic DNA followed by hybridization with Vβ and Cβ probes suggests that there are 4-6 individual VDJC clusters in the shark genome [143]. This clustered organization contrasts the V_n D J C D J C translocon organization found in mice and humans [132]. A study of a spleen cDNA library suggests that not all of the clusters are actively transcribed [143]. A study of variability in the shark TCR Vβ segments is carried out in chapter 2 using Shannon Entropy analysis.

1.3.1.6.2 The clearnose skate

Again, only a small sample of cDNAs were investigated in the skate and additional families of segments are expected to be present [144]. In 18 clones, 6 Vβ, 4 Jβ, 2 Dβ and 2 Cβ segments were characterized. 2 Cα, 4 Vα and 6 Jα segments were found as well, suggesting that significant combinatorial diversity is found in cartilaginous fishes [144].
Therefore the makeup of the TCR β locus in vertebrates is quite plastic. This raw genomic repertoire of segments provides much of the variability, that allows vertebrates to recognize such a great range of antigens. The makeup and variability of the genomic repertoire of *Xenopus* is described in chapter II. The *Xenopus* model is an attractive one

1.3.1.6.2 The clearnose skate

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1.3.2 The *Xenopus* model

1.3.2.1 Pivotal evolutionary position

Xenopus, an anuran amphibian, is a representative descendent of the first terrestrial vertebrates (tetrapods)(as reviewed in [145]). *Xenopus* has become an important developmental and comparative model for the study of the immune system due to this pivotal position in vertebrate evolution. As such, it is one of the best studied of the poikilothermic vertebrates and many genes and molecules involved in immunity have been defined in *Xenopus* (table 1.1) and inbred and clonal (gynogenetic) lines of *Xenopus* as well as lymphoid cell lines are available [263-265]. Although the organization and makeup of the *Xenopus* adaptive immune system closely resembles that of other vertebrates, *Xenopus* does vary from mammals and birds in several key respects.

1.3.2.2 Developmental model

First, *Xenopus* is free-living from day 2 post fertilization, and is therefore more exposed to the outside world than some reptiles, birds and mammals which are protected during embryonic life by an egg or womb. The ontogeny of T cells may differ somewhat due to these differences in life cycle.

Second, the *Xenopus* lifecycle is articulated around the profound changes of metamorphosis when the tadpole transforms into a frog. Because metamorphosis is a time when many "new" adult antigens are expressed, there are mechanisms which protect these newly emerging tissues from attack by the tadpole immune system [146]. Indeed, at metamorphosis the tadpole lymphoid organs are reshuffled, the lymphocyte repertoire is destroyed and a new adult lymphocyte repertoire is produced (a second histogenesis) [147] as reviewed in ([1]).

Third, there are unique developmental differences in the expression of genes involved in immunity between adults and tadpoles as well. Unlike adults, tadpoles do not express MHC class Ia or Ib in the thymus [159]. Both adults and tadpoles express class II, although with a different tissue distribution (see section 1.2.1.3.2).

Thus, the *Xenopus* immune system provides a unique selection environment for T cells at each life stage. *Xenopus* is the only animal studied thus far that shows this larval/adult dichotomy in MHC class I expression and permits a unique opportunity to study T cell selection on different levels of MHC class I in a natural, non-manipulated system.

1.3.2.3 Technical advantages

The issue of what constitutes a locus or an allele is greatly simplified by using gynogenetic clones of interspecies hybrids between *X. laevis* and *gilli*. The species specific alleles can also be recognized as belonging to either the *laevis* or *gilli* genome, thus simplifying many of the genetic complications working with a tetraploid organism brings. Female 'LG' hybrids of *Xenopus laevis* and *gilli* produce both haploid and diploid eggs. The diploid eggs are product of endoreduplication during meiosis and can be activated by adding UV irradiated sperm which cannot contribute their damaged DNA to the zygote. Activated eggs develop into gynogenetic clones of their mother (reviewed in [148]). LG clones therefore provide an excellent uniform genetic background for experimentation.

In ontogeny, there is free access to the early stages of development. This, and the transparancy of the tadpoles, make manipulations such as thymectomy possible.

1.3.3 An overview of T cell immunity in *Xenopus*

Here I will review what is known about T cell immunity in *Xenopus*. This is not meant as a comprehensive review, but rather an introduction to the parts of the system that will be refered to in this work.

1.3.3.1 *Xenopus* TCR and MHC

1.3.3.1.1 TCR

Previously, 10 Vβ and 10 Jβ segments were found associated with 2 Dβ segments and a single constant region in *Xenopus* [149]. *Xenopus laevis* Cβ region is unique in that it lacks a site for N-glycosylation. The only other Cβ region to share this characteristic is the axolotl Cβ1-3 region (see section 2.3.1). Recombination signal sequences (RSS) were found like those of other studied vertebrates which suggested that the mechanism of recombination for the TCR was conserved at the amphibian level.

1.3.3.1.2 MHC

Classical class I MHC (class Ia) molecules were first identified in *Xenopus* via alloantisera. Immunoprecipitation showed a complex made up of a large 40-44 kDa protein, presumed to be the class I heavy chain and a small 13 kDa non-glycosylated chain which was assumed to be the β_2m subunit [150]. The polymorphism of these determinants was found to be genetically linked to the MHC. *Xenopus* class Ia sequences are very polymorphic within the population [151]. Using several alloantisera, it was noted with some surprise that class Ia molecules are not detected in tadpoles until just before the onset of metamorphosis [152, 153]. Because the alloantisera were not certain to recognize denatured heavy chains, the monoclonal to the class I heavy chain was used to confirm these results. However, the monoclonal antibody to the class I heavy chain only recognizes class I molecules from several specific haplotypes and can therefore only be used in experiments employing specific lines of frogs [153]. Experiments in which metamorphosis is chemically blocked show that, unlike class II, class I expression levels gradually increase over time, independent of metamorphosis [154]. At day 27 (stages 53-54) surface MHC class I cannot be detected on splenocytes, erythrocytes and

58) and day 53 (stage 60), respectively [154, 155]. By metamorphic climax, significant levels of class Ia and Ib become expressed and adult levels of surface class I MHC expression are present by 2 months post-metamorphosis (as reviewed in [36]). However, adult frogs do express class I molecules on all of their tissues including thymus epithelial cells [153]. This dichotomy of expression could affect the selected T cell repertoire. Because the majority of cytotoxic T cells require classical MHC class I molecules for selection and the remaining CTLs require non-classical MHC class I molecules it could be predicted that a tadpole CTL repertoire might have a very different TCR repertoire than the adult.

Xenopus possesses only a single MHC class I locus, unlike all other studied vertebrates. [156]. Chickens, rabbits and dogs possess more than one locus but only one active or predominantly expressed locus. Analysis of Southern transfers with domain specific probes and recent chromosomal *in situ* experiments show that both class Ia and Ib are diploidized in polyploid *Xenopus* species (see discussion) and are found on the same acrocentric chromosome [156, 157].

Nine subfamilies of non-classical (class Ib) genes have been isolated in *Xenopus* and all map to a distinct non-MHC linked region [158, 159]. Although genetic studies found the XNC to be unlinked to the MHC proper recent chromosomal *in situ* data has shown that both class II and Ib are present on the same acrocentric chromosome [157]. Because class I and II are closely linked based on a very low incidence (~1%) of recombinants in family studies, we can conclude that class Ia is located on the same acrocentric chromosome. *Xenopus* non-classical (XNC) molecules, have the same basic structure of all class Ia molecules but do not contain all of the amino acid (aa) residues needed for peptide binding in the groove [158]. They are also much less polymorphic than *Xenopus* class Ia genes [159]. In adult frogs, XNC isotypes are differentially expressed in lung, intestine, spleen and in thymus but not in liver, colon or muscle. Unlike other studied vertebrates, Northern blot analysis failed to detect any expression of XNC during tadpole life [159].

In mice, a small population of CD8$^+$ T cells are selected on non-classical MHC molecules [160]. However, in *Xenopus* tadpoles non-classical molecules clearly cannot fulfill the role of class Ia in T cell differentiation. The expression pattern of XNC genes is very similar to that of class Ia but much more stringent in late tadpole life where class Ia is expressed at a low level but XNC cannot be detected [159]. The lack of class I expression is therefore controlled at the two loci at chromosomal regions quite distal to each other, therefore suggesting an evolutionary selection for limiting all class I expression during tadpole life [159].

1.3.3.2 *Xenopus* immune function during development

Xenopus $\alpha\beta$ T cells differ from those of mammals and chickens mainly in that they are active during larval life. Mammals and chickens are well protected during fetal life and benefit from maternal antibodies. *Xenopus* are free-living from 2 days post-fertilization (dpf) and must quickly upregulate both innate and adaptive immune responses.

1.3.3.2.1 Thymus

Xenopus tadpoles hatch from their egg casings at 2 days post fertilization (dpf). On day 3 or 4, the thymus, the first lymphopoietic organ in *Xenopus* buds off from the second pharyngeal pouch. The twin thymi are colonized on day 4 or 5 by precursor lymphocytes derived from the ventral and dorsal lateral plate mesoderm and more specifically from blastomere C4 [161-165]. The thymic epithelium begins to express MHC class II molecules at this time independently of the colonization of the thymus by T cell precursors [166]. Unlike other vertebrates studied, classical MHC class I molecules are not expressed in the tadpole thymus. Between stages 48 and 49 (~7-12 dpf) T cell precursors proliferate in the thymus, a cortex and medulla architecture becomes apparent, and the organ becomes vascularized [167, 168]. During this time, cells in the cortex begin to undergo apoptosis suggesting that the process of selection has started [168]. The thymus continues to grow to a maximum cellularity of ~ 2 x 10^6 thymocytes before the onset of metamorphosis [169], when the majority of T cells undergo corticosteroid induced apoptosis [170].

Removal of the thymus (thymectomy, Tx) on day 4 post-fertilization (stage 45 ~ 4 dpf) completely eliminates T cell dependent functions such as acute allograft (graft from an animal expressing at least one different MHC antigen) rejection, MCR. Additionally, reactions to T cell dependent antigens and mitogens are absent and an IgY (homologue of mammalian IgG) humoral response cannot be mounted [171, 172]. Such humoral responses require help from class II restricted T cells [173, 174]. Tx at 7-8 dpf does not always eliminate these responses [175-178]. Functional T cells therefore begin to leave the thymus around days 7 to 8 when the thymus contains less than 300 cells (as reviewed in [178]; see chapters 3 and 4). Staining with several monoclonal antibodies against *Xenopus* T cell markers confirms the appearance of T cells in the periphery. Yet experimentally, tadpole T cells cannot provide the same level of T cell help as do adult cells [179]. Thus, it was concluded that there is a lack of T cell help in tadpoles (see section 7.5.1.2).

1.3.3.2.2 Spleen

The spleen, a secondary lymphopoietic organ in *Xenopus*, is first histologically distinguishable on day 11 or 12 post-fertilization, and is immediately colonized by B and T cells (as reviewed in [145] and [178]). During larval life it is essentially lymphopoietic. The spleen continues to grow in size until it reaches a maximum number of $5x10^5$ lymphocytes during larval life (as reviewed by [180]) In the adult the spleen contains areas of erythropoiesis (red blood cell production) red pulp and areas with T and B lymphocytes or white pulp [162]. As is the case in other ectothermic vertebrates, the *Xenopus* spleen does not contain lymphoid follicles, germinal centres or lymph nodes analogous to those found in mammals and birds (figure1.8) [181] (reviewed in [182]). The spleen continues to grow in size until it reaches a maximum number of $\sim 5 \times 10^5$ lymphocytes just before the onset of metamorphosis (reviewed in [180]).

1.3.3.2.3 The ontogeny of MHC expression

In studying the ontogeny of $\alpha\beta$ T cells, one must also take into account the expression of the TCRs ligands, the MHC class I and II molecules. Only one classical MHC locus exists in *Xenopus*, in contrast to the 1-3 polymorphic loci present in mice, humans and all other characterized animal models [156]. Chicken, dogs, and rabbits possess multiple class Ia loci but only one locus is predominantly expressed ([183, 184] as reviewed in [116]), leading to a very similar situation to *Xenopus*. The low number of loci in individuals suggests that more than 3 class I loci is deleterious. *Xenopus* is the only animal studied thus far that shows this larval/adult dichotomy in MHC class I expression. Thus, the tadpole immune system provides a unique selection environment for T cells.
Tadpoles do not express surface MHC class Ia or Ib molecules until very late in tadpole life. Experiments in which metamorphosis is chemically blocked show that, unlike class II, class I expression levels gradually increase over time, independent of metamorphosis [154]. At

day 27 (stages 53-54) surface MHC class I cannot be detected on splenocytes, erythrocytes and thymocytes [154]. Low surface expression of class I molecules is detected in splenocytes, erythrocytes and thymococytes at day 34 (stage 55-56), day 46 (stage 57-58) and day 53 (stage 60), respectively [154, 155]. By metamorphic climax, significant levels of class Ia and Ib become expressed and adult levels of surface class I MHC expression are present by 2 months post-metamorphosis (as reviewed in [36]). Despite the lack of surface class I expression in the thymus, CD8$^+$ T cells do develop during tadpole life implying selection on class I MHC molecules. Despite the lack of surface expression, Northern blotting for classical class I detects mRNA expression in the lungs, gills, pharynx and gut. Most tissues that express MHC Ia mRNA are either destroyed or undergo significant restructuring at metamorphosis [36]. Perhaps CD8$^+$ T cell selection is mediated by a very small number of class Ia protein molecules on the cell surface. Selection on known non-classical MHC Ib molecules does not provide an alternative explanation for the selection of CD8$^+$ T cells in tadpoles for non-classical MHC class Ib mRNA is not detected by Northern blotting during tadpole life [36].

Class II MHC molecules are expressed by both tadpoles and adults, although with a different tissue distribution. Class II expression is first detected in the tadpole thymus epithelium by immunofluorescence 4-5 days after fertilization [166, 185]. Later in tadpole life, class II heterodimers are expressed on B cells, macrophages and the spleen reticulum, as well as on occasional patches of cells in the skin and gut. Macrophages in the liver, skin, and brain are class II positive from the midlarval stages onwards. The epithelial surfaces of the gill and pharyngobuccal cavities express class II MHC even on surfaces where these epithelia are exposed to the outside environment. Thus, during tadpole life, class II expression is most concentrated in the tissues which make contact with the outside environment as opposed to such class II epithelia in mammals, which tend to contact the internal environment [185].

In adults, class II is expressed in the thymic epithelium, in the skin (thought to be Langerhans-like cells) and on the surface of B and T cells as well as macrophages. The expression on T cells and the lack of expression on the adult gut epithelium is what sets adult expression apart from that of tadpoles [185].

Class II MHC is not expressed on T cells in mice. However, T cells in other studied species including humans, dogs, horses, dolphins, pigs, rats, axolotl and cats do express surface class II MHC molecules, especially upon activation. [36, 186-194]. The expression of class II MHC on adult *Xenopus* T cells is therefore not exceptional.

However, what is exceptional is that class II expression is only found on adult T cells in *Xenopus*. Therefore, class II expression in T cells is differentially controlled in tadpoles and in adults.

1.3.3.2.4 Evidence for selection

Positive selection of T cells in *Xenopus* was demonstrated using MHC mismatched chimeras or Tx animals which were then implanted with MHC mismatched thymuses [174, 195-197]. This forces T cell precursors of one MHC background to be selected on MHC molecules from a different haplotype. After such cells move into the periphery they should, in the case of absolute MHC restriction, only recognize peptides presented by the selecting MHC and ignore peptides presented by the host MHC. In some cases restriction is not absolute. However, grafts differing by at least one MHC antigen are rejected in adult *Xenopus* therefore showing an MHC restriction to the MHC of the educating thymus [198]. Tadpole T cells can respond to cells from tadpoles differing by at least one MHC antigen but due to technical difficulties this experiment was never studied in tadpoles with tadpole grafts.

There is ample evidence for peripheral tolerance in *Xenopus*. *Xenopus* tadpoles, especially around the time of metamorphosis, can be made tolerant to MHC mismatched embryonic and adult tissues by grafting. This tolerance persists through metamorphosis into adulthood. *In vitro* experiments have shown that T cells reactive to embryonically grafted tissue are present but *in vivo* tolerance is maintained presumably by the downregulation/anergy of such cells *in vivo* (as reviewed in [199]). The function of the *Xenopus* thymus in selection of T cells and the mechanisms of peripheral tolerance, seem similar to that of birds and mammals. This supports the use of *Xenopus* as a model for selection and to measure the TCR repertoire in an animal which differs from mammals in terms of MHC expression patterns, size, ambient temperature and lifestyle.

1.3.3.3 Metamorphosis

At metamorphosis, the majority of lymphocytes found in the thymus, spleen, and liver undergo corticosteroid induced apoptosis ([169]; as reviewed in [170, 200].). It has been hypothesized that this occurs to avoid the destruction of the new tissues expressing adult antigens. The loss of lymphocytes at metamorphosis results in a period of immunosuppression, before lymphoid precursors again enter the thymus and production of adult thymocytes begins (reviewed in [200]).

1.3.3.4 TdT differences

Like birds and mammals before hatching or birth, *Xenopus* tadpoles lack significant TdT expression until metamorphosis [201-203]. This limits the addition of nucleotides at the VDJ joints and thus the length and diversity of the CDR3 junctions in tadpole immunoglobulins [202]; and as reviewed in [203]). In *Xenopus*, there is very little TdT mRNA during midlarval stages and only during the metamorphic climax does one first see appreciable increases in expression [202]. Expression of TdT during early embryonic life has been reported only in the Rainbow trout [182]. The junctional diversity of CDR3 regions of the TCR are also expected to be limited by the lack of N nucleotide additions due to the lak of TdT expression during tadpole life. In this study we will compare the length of tadpole and adult CDR3 junctions. [201, 204].

1.3.3.5 B and T Cell memory persists through metamorphosis

Although, at metamorphosis, most tadpole lymphocytes are destroyed, there is good evidence that some memory cells do persist into adulthood. Adult frogs that were primed with DNP-KLH or foreign red blood cells while tadpoles could respond to these antigens with accelerated kinetics of a secondary response. Also antibodies raised in tadpoles to a specific hapten could be found again (via isoelectric focusing) in adult frogs after metamorphosis. ([152, 205] [207, 208, 209, 210, 263] and as reviewed in [200]). Similarily, tadpoles which rejected alloogenic skin or irradiated tumour cells (a response mediated by T cells) could reject the same allograft as adults with the kinetics of a secondary response [206-210] or as reviewed in [200].

Xenopus provides us with an opportunity to study the development of T cells as well as the diversification and selection of the T cell receptor β chain repertoire in a system which is a natural variation on the theme of the vertebrate adaptive immune system.

1.5.3 Thesis goals

The goals of this thesis work are fivefold.

1) The genomic repertoire of V, D, J and C segments is described in *Xenopus laevis* and *gilli*, haplotypes of LG hybrids, with special emphasis on the variability of those segments (chapter 2).
2) The reactivity of a polyclonal serum which recognizes *Xenopus* CD3ε is defined. The tissue distribution of CD3ε expression in adults as well as in solid tumours and the frog thymoma cell line B3B7 is described (chapter 3).
3) The ontogeny of *Xenopus* T cells is addressed, including the spatial and temporal onset of rearrangement at the TCR β locus, as well as the nature and the diversity of these rearrangements (chapter 4).
4) A study of the repertoire of V-D-J rearrangements at the TCR β loci of tadpoles and adults is carried out. In the course of these studies, the normal thymic (unselected) and peripheral (selected) repertoires are characterized in both tadpoles and adults (chapter 5).
5) The theoretical implications of temperature changes on T cell selection, development and activation in poikilotherms will be addressed in light of recent data from homeothermic systems and discussed in the context of current models (chapter 6).

The major findings and conclusions of this work are summarized at the end of each chapter and are discussed further in chapter 7.

No Text

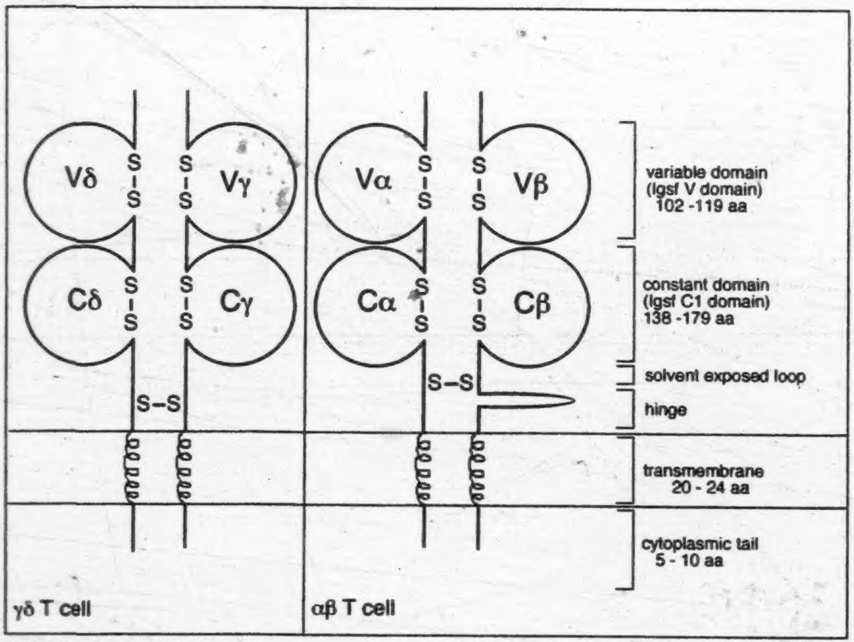
marker	**reference**
CD3 γ/δ	77
CD3ε*	78
TCRβ	149
CD8*	260
RAG1	217
RAG2	217
TdT	202
CD5*	218
CTX	219
CD45	220
LMP-7	221
LMP-2	254
TAP1	259
TAP2	226
MHC Ia	150, 156
MHCIb	158
MHC II	256, 257
IgL	213, 222, 223
IgH	211, 212, 214, 215, 216
XT-1*	255
Factor B	224
C3	261
C4	225
β_2m	BI477286
IL16	BG555391
RANTES	BG656988
calreticulin	BI348196
perforin	BI477295
tapasin	BG017752
LY-9?	BI477282
NKp44?	BI477258

Table 1.1 Markers of the immune system in *Xenopus*

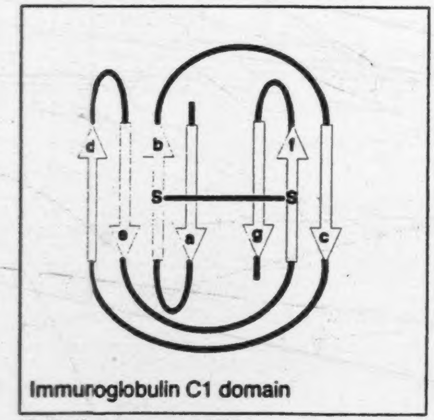
A summary of *Xenopus* genes which have been cloned or the product of which have been identified by immunological means. Markers for which antibodies are available but which have not been cloned are marked with an "*". Accession numbers are given for newly cloned genes that have not been experimentally confirmed or formally published.

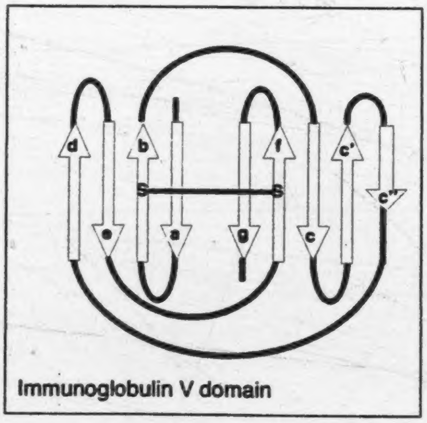
Figure 1.1 Schematic Diagram of the Domain Structure of T Cell Receptors

Both γδ and αβ TCRs are composed of two disulfide linked chains. Each chain contains two immunoglobulin domains as well as a hinge, transmembrane, and a cytoplasmic domain. The membrane proximal and distal domains are immunoglobulin superfamily domains of the C1 and V types respectively. Schematic diagrams of these domains are given in (b) and (c). Immunogobulin domains are formed by two antiparallel β pleated sheets held together by an intrachain disulfide bond. The V (variable) domain contains two more strands (c' and c") than the C1 (constant) domain. In mammalian TCR β chains, the loop formed at the transition from the f to g strand of the constant region is much longer than in other studied vertebrates. This region is often referred to as the solvent exposed loop and it is not required for the development or function of normal αβ T cells in mice [228]. The hinge region is encoded by a small exon of only 6 aa containing the cysteine involved in the interchain disulfide bond. The transmembrane domains of all of the TCR chains encode positively charged aa which are involved in interactions with the CD3 complex that is associated with the TCR and is necessary for signaling through the receptor. The cytoplasmic tails of TCR chains are very short and contain no signaling domains. This figure has been modified from [229] and [230].


Vδ
Vγ
Cδ
Cγ
S-S
Vα
Vβ
Cα
Cβ
S-S
S
S
variable domain
(Igsf V domain)
102 -119 aa
constant domain
(Igsf C1 domain)
138 -179 aa
solvent exposed loop
hinge
transmembrane
20 - 24 aa
cytoplasmic tail
5 - 10 aa
γδ T cell
αβ T cell

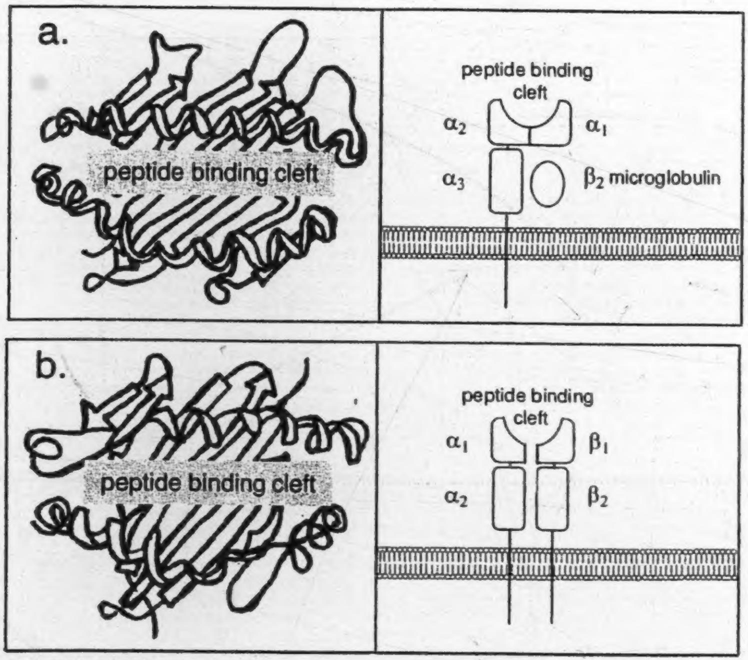
Modified from Abbas et al, 1994.


d
b
f
S
S
e
a
g
c
Immunoglobulin C1 domain


d
b
f
c'
S
S
c''
e
a
g
c
Immunoglobulin V domain

Modified from Janeway et al, 1999.

No Text


a.
peptide binding cleft
peptide binding cleft
α2
α1
α3
β2 microglobulin
b.
peptide binding cleft
peptide binding cleft
α1
β1
α2
β2

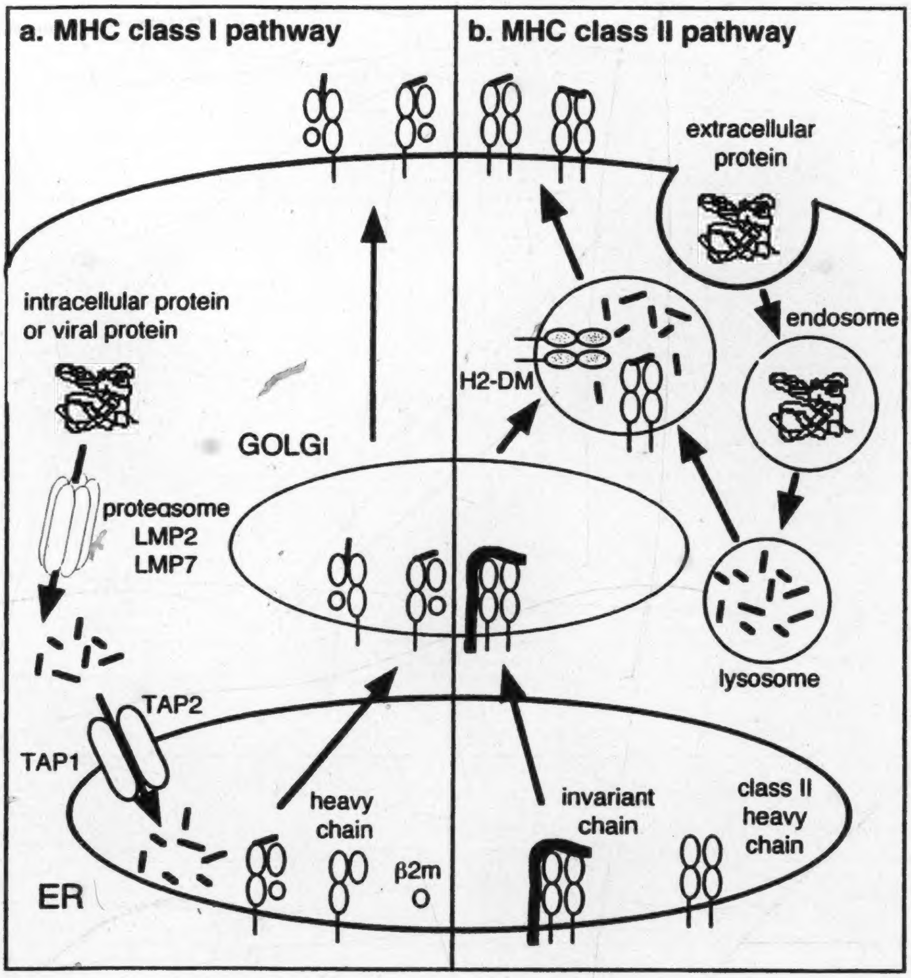
Redrawn from Janeway et al, 1999.

Figure 1.2 The Structure of MHC Class I and II Molecules

a) Class I MHC molecules are composed of a heavy chain with three extracellular domains (α1, α2, α3) complexed with β_2-microglobulin (β_2m). b) Class II molecules are heterodimers composed of an α and β chain each with two extracellular domains (α1, α2; β1, β2). In class I molecules the α1 and α2 domains form a peptide binding cleft which is composed of two α helices over a β pleated sheet. In class II molecules α1 and β1 form a very similar peptide binding cleft. (figure modified from [230]).

Figure 1.3 Antigen processing and presentation

Class I MHC molecules present peptides derived from intracellular sources (endogenous proteins or proteins derived from viral or bacterial intracellular pathogens). These proteins are processed into short peptides by the proteasome and transported to the lumen of the ER by the TAP (transporter associated with antigen processing) complex. Here peptides are loaded into the peptide binding grooves of class I heterodimers and then are transported through the Golgi and to the cell surface. b) Class II molecules present peptides derived from extracellular sources (serum or pathogen proteins etc.). Proteins are endocytosed and this endosome fuses with a lysosome containing enzymes which degrade the protein into small peptides. The class II heterodimer assembles in the ER but its peptide groove is blocked by the invariant chain until its endosome fuses with a special endosome containing both peptides and a molecule called H2-DM. H2-DM helps displace the invariant chain and with the chain gone, class II molecules are loaded with peptides and then move to the cell surface. Figure adapted from [35] and [230].


a. MHC class I pathway
b. MHC class II pathway
extracellular protein
intracellular protein or viral protein
endosome
H2-DM
GOLGI
proteasome LMP2 LMP7
lysosome
TAP2
TAP1
heavy chain
invariant chain
class II heavy chain
β2m
ER

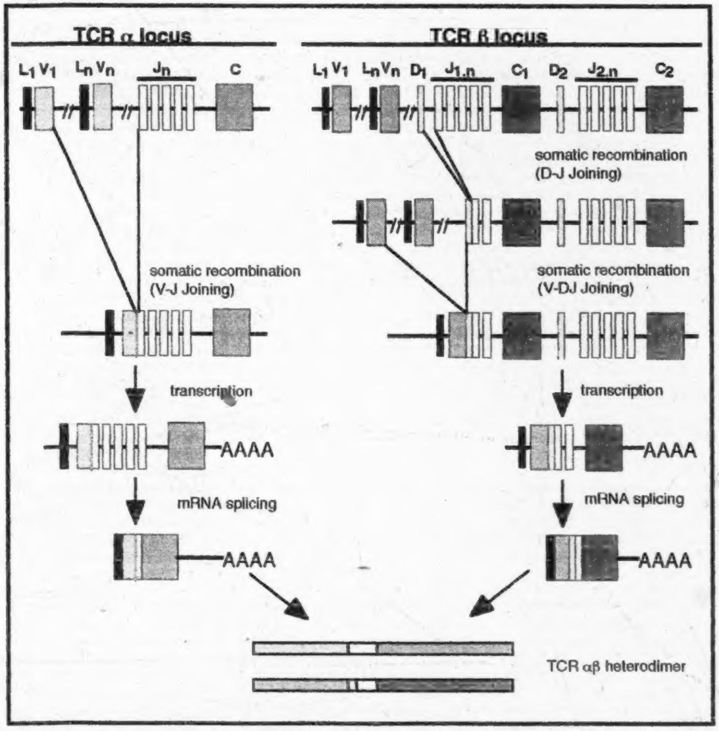

TCR α locus
TCR β locus
L1 V1
Ln Vn
Jn
C
L1 V1
Ln Vn
D1
J1.n
C1
D2
J2.n
C2
somatic recombination
(D-J Joining)
somatic recombination
(V-J Joining)
somatic recombination
(V-DJ Joining)
transcription
transcription
AAAA
AAAA
mRNA splicing
mRNA splicing
AAAA
AAAA
TCR αβ heterodimer

Fig. 1.4 VDJ recombination

A process of somatic DNA recombination creates a unique receptor in each T and B cell. The β locus contains an array of V, (variable), D (diversity), J (joining), and C (constant) regions. In a process requiring both RAG1 and 2, a D segment is randomly joined to a J segment. This cut is imprecise and is followed by removal of nucleotides by a exonuclease and the addition of nucleotides by TdT resulting in additional diversity at the join (see text). Next, a Vβ segment is rearranged to the DJ. Processing of the mRNA derived from the somatically rearranged genomic locus leads to the final TCR β transcript. Similarly, the α locus (which lacks D segments) undergoes VJ rearrangement and is transcribed and processed. The red shown on the figure at the α VJ junction represents the extra variability generated at the join. L represents the leader 5' of each V.

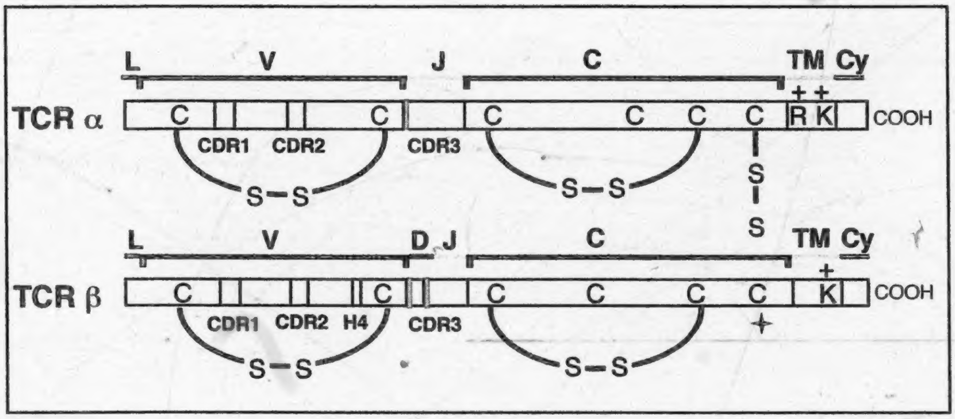

L
V
J
C
TM
Cy
TCR α
C
CDR1
CDR2
C
CDR3
C
C
C
C
R
K
COOH
S–S
S–S
S
S
L
V
D
J
C
TM
Cy
TCR β
C
CDR1
CDR2
H4
C
CDR3
C
C
C
C
K
COOH
S–S
S–S

Figure 1.5 The structure of the T cell receptor heterodimer. Both the α and β chains consist of two immunoglobulin domains followed by a short membrane proximal connecting peptide, a transmembrane (TM) region containing positively charged residues and a short cytoplasmic tail (CYT). The regions are as follows: L, leader; V, variable; D, diversity; J, joining; and C, constant; CDR, complementarity determining region; and H4, fourth hypervariable region. Note (*) that the β chain cysteine involved in the interchain disulfide bond is absent in the teleosts. Mammals also have a 14 aa solvent exposed loop between the f and g strands of the β chain. Note that CDRs 1, 2 and H4 are genomically encoded by the variable region. Figure adapted from [Davis, 1999].

Figure 1.6 The interaction of the TCR complex with a peptide-MHC complex

A crystal structure depicting the interaction between an αβ T cell receptor and a class I molecule complexed with peptide. A) The binding surface of the TCR where α or β 1, 2 and 3 represent CDRs 1,2 and 3 respectively. HV4 indicates the position of the fourth hypervariable region. Note how the CDRs from both the α and β chains come together to form the binding surface C) The binding surface of the peptide-MHC complex, showing the α1 and 2 domains of the class I molecule and three important contact residues in the peptide (yellow). B) An overlay of A and C showing the orientation of the interaction. D) A ribbon diagram depicting the same interaction from another perspective. Figure reprinted from [62] by permission of C. Garcia.

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Figure 1.7 The T cell receptor complex

(a) The TCR complex is composed of either an αβ or γδ heterodimer and CD3ε, CD3 γ/δ and CD3 ζ chains. In mammals (b), a duplication has resulted in distinct but related γ and δ molecules. Charged residues in the transmembrane allow the chains to associate. Signaling is dependent on the immunoreceptor tyrosine-based activation motifs (ITAMs) (in blue) located in the cytoplasmic domains of the CD3 molecules. Figure redrawn from [74].

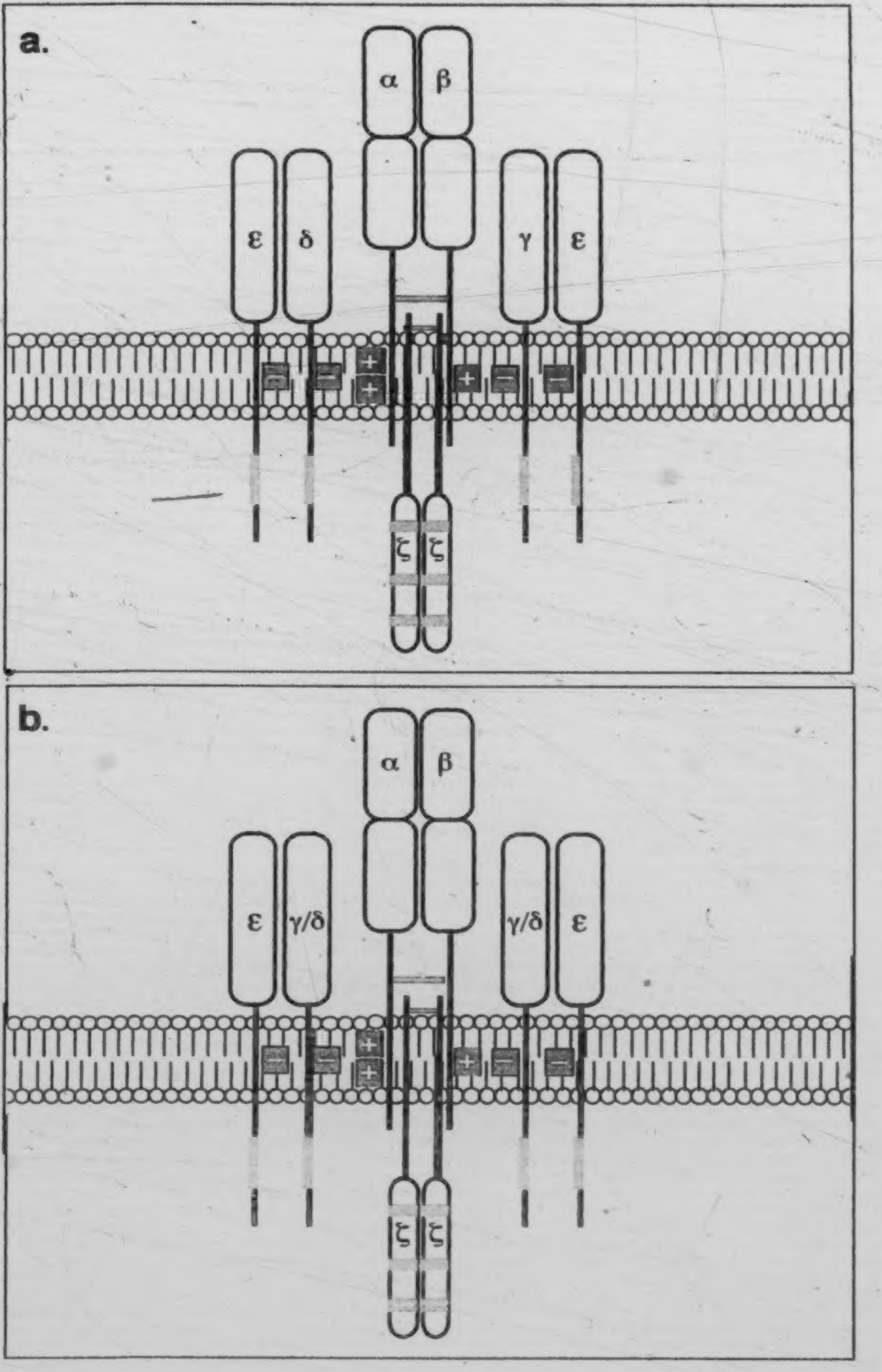
a.
α
β
ε
δ
γ
ε
+
+
+
−
−
−
−
ζ
ζ
b.
α
β
ε
γ/δ
γ/δ
ε
+
+
+
−
−
−
−
ζ
ζ

Figure 1.8 An evolutionary view of vertebrate immune systems

All jawed vertebrates (gnathostomes) possess the parts of an adaptive immune system whereas the jawless vertebrates (agnathans) do not. “+” refers to the presence and “—“ to the absence of an organ or a gene. RAG refers to the presence of both RAG 1 and 2. The letter codes for lymphoid organs are: T., thymus; S, spleen; L, liver; G, GALT; LN, lymph nodes; B, bone marrow; and GC, germinal centers. “?” represents cases where a molecule has not been cloned but there is evidence that it should be present. “s” refers to the fact that the bone marrow in amphibians is not as important a site of lymphoid development as in other species. “f” refers to the *Xenopus* fetal liver which is hematopoietic. Organisms which illustrate a group may not be fully representative of the entire group. References taken from ([1, 42, 138-144, 162, 182, 231-253]).

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Chapter 2

Variability of segments that make up the *Xenopus* TCR β chain

2.0 Variability of segments making up the *Xenopus* TCR αβ chain

2.1 Introduction

The immense diversity of T cell receptors produced in an individual starts from a repertoire of genomically encoded TCR β segments. From this raw material, a process of somatic DNA recombination pieces together a variable (V), diversity (D), and joining (J) region to create a unique TCR β chain. Splicing of the VDJ "gene" to the constant domain (C) during mRNA processing leads to the final transcript for the receptor. The VDJ join is imprecise, with bases being added or removed at the points of joining, resulting in an extremely variable, somatically produced region called the third complementarity determining region (CDR3). Two other regions of variability, CDRs 1 and 2, are genomically encoded in the Vβ segments. The number and variability of genomically encoded TCR β segments is important for the TCR β repertoire because it determines the maximum number of unique T cell receptors which can be produced in an individual.

In mice, humans and chickens this diversity has been well defined by genomic sequencing of the loci. The human TCR β locus contains 65 Vβ segments, 47 of which are functional, encompassing 30 Vβ families. Families are defined by 75% DNA homology. These V elements are found upstream of 2 D-J-C clusters, each containing a single Dβ segment, 6-7 Jβs and a single Cβ [1, 2]. The genomic organization of the mouse locus is homologous to that of humans. 25 functional Vβ segments, that can be grouped into 20 families, are associated with 2 D-J-C clusters, each made up of one Dβ , 5 functional Jβs and a single constant region [2, 3]. As is the case for the genes of the MHC, chickens possess a decidedly minimalist TCRβ locus, containing only 2 Vβ families composed or 4 to 5 members each, 3 Jβ segments and a single Dβ and Cβ segment [4, 5].

Information concerning the diversity and genomic organization of TCR loci in poikilothermic vertebrates is quite limited. Partial information is available for many species but TCR β segments have not been exhaustively cloned from any poikilothermic vertebrate. Here I will only consider the best characterized models, the horned shark (*Heterodontus francisi*) and Mexican axolotl (*Ambystoma mexicanum*). For a review of TCR β chains in other poikilothermic vertebrates, I direct the reader to section 1.3.1. In the horned shark, *Heterodontus francisi,* there exist at least 4 Cβ segments, 7 Vβ families, a single Dβ, and 18 Jβ segments contained in several V-D-J-C clusters [6]. In the axolotl, a urodele amphibian, 4 Cβ segments have been found along with 13 Vβ, 4 Dβ and 13 Jβ segments [7-12].

An exhaustive cloning of *Xenopus* TCR β segments was carried out to supplement the subset of TCR β sequences that has previously been cloned from *Xenopus* [13]. This was necessary to give a realistic view of the genomic TCR β diversity present in a poikilothermic vertebrate and to provide a knowledge of the available TCR segments for further work on the onset of TCR β recombination during ontogeny (see chapter 4) and for a study of the expressed TCR β repertoire in *Xenopus* (see chapter 5).

TCR β segments were isolated from clonable LG3 and LG15 *laevis/gilli* interspecies hybrids. [14]. The use of such interspecies hybrids has distinct advantages over other poikilothermic models where the analysis of the TCR β locus is complicated by the use of outbred animals making the distinction between an allele and a locus difficult. Because the *laevis* and *gilli* constant regions differ in sequence and the grand majority of TCR β rearrangements occur within a locus (in *cis*) rather than between TCR β loci located on different chromosomes (in *trans*) [15, 16], we can determine which segments are associated with the *laevis* or *gilli* constant regions and thus build a picture of each locus. Although this is not a formal sequencing of the locus we are confident that this "implied" genomic/locus information will be confirmed by genomic sequencing.

The variability of these segments is measured for the first time using Shannon entropy analysis showing *Xenopus* Vβ segments are as variable as those of mammals or of axolotl or shark.

2.1 Materials and methods

2.1.1 Animals

LG3 and LG15 animals were produced by gynogenesis as previously described [14] and staged according to the Nieuwkoop and Faber table [17]. Haploid progeny, produced by activation of eggs with UV irradiated sperm, were collected at 3-4 dpf and processed as previously described [18]. LG3 and LG15 clones are respectively of haplotypes bd and ac at the class I MHC loci but share a common TCR β locus [13, 19].

2.1.2 Generation of 5' RACE libraries, amplification and subcloning

RNA was derived from spleen and thymus using Trizol (GibcoBRL) according to the manufacturer's instructions except that 15μl of water per 1ml Trizol was added to the initial homogenization step and that glycogen was used as a carrier in all precipitations. 450-500 ng of total RNA was used to generate 5' RACE libraries using the Smart RACE kit (CLONTECH) according to the manufacturers instructions except that random hexamer primers were added to prime the first strand synthesis in addition to the given anchored poly-T primer and that the adapter oligonucleotide was added after 2 minutes of cDNA synthesis. 1μl of template RACE cDNA was amplified with a sense adapter specific primer (CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT) and an antisense Cβ primer (ATTCTTGGTGAGGCTGAGG CGGCTGC) using Li^+ based dNTPs, Taq DNA polymerase and 10X PCR buffer (Perkin-Elmer) in a volume of 50μl. PCR was carried out on a Techne thermocycler as follows: 5 cycles of 94°C 10sec, 68°C 10 sec, 72°C 2 min, 5 cycles of 94°C 10sec, 65°C 10 sec, 72°C 2 min, and finally 25 cycles of 94°C 5 sec, 63°C 10 sec, 72°C 2 min followed by 10 minutes at 70°C. 1 μl of this initial reaction was reamplified in 50μl volume as described above but employing a nested sense primer in the adapter region (AAGCAGTGGTAACAACGCAGAGT) and the same Cβ antisense primer. The cycling conditions for this second PCR were 94°C 5 sec, 63°C 10 sec, 72°C 1 min for 25-30 cycles, followed by 10 minutes at 70°C. PCR products were then separated on a 1% agarose gel and bands larger than 450 bp were purified using Geneclean (BIO 101). This population of products was then ligated into the PCR II dual promoter plasmid using the TA cloning kit (Invitrogen) and used to transform appropriate *E. coli* strains.

2.1.3 PCR primers and sequence

Sequencing was carried out on both ABI and LI-COR based systems. Sequences were then compared to a local sequence database using software developed by Stefan Meyer based upon ther BLAST algorithm. Sequence alignments were generated using either the Lasergene multi-sequence alignment tool (Megalign) or CLUSTAL using a PAM 250 matrix. Genbank accession numbers for previously submitted sequences can be found in the series U60424 to U60436 and U75994.

2.1.4 Southern transfer analysis

10 μg of LG3 and LG15 erythrocyte genomic DNA was digested to completion with EcoRI, PstI, BamHI, and HindIII respectively, as recommended by the enzyme supplier, migrated on a 0.85% agarose gels, and transferred to Zeta-Probe membranes (BIO-RAD) via alkaline transfer as previously described [13]. The filters were UV crosslinked and prehybridized as previously described [13]. Low stringency hybridizations were carried out with either entire V or C domain probes at 55°C for 72 hours and then washed at 56°C in 2x SSC and 0.1% SDS for 30-60 min and autoradiographed using BIOMAX MR films (KODAK). High stringency hybridizations were carried out at 65°C for 16-24 hours and then washed at 65°C in 0.1x SSC and 0.1% SDS for 30 min and autoradiographed using BIOMAX MR films (KODAK). Genomic DNA was isolated from haploid LG15 embryos [18] and digested to completion using NcoI or EcoRI and then subjected to the same Southern transfer and probing procedure described above.

2.1.5 Variability analysis

Alignments were carried out for *Xenopus*, shark, axolotl, mouse and human Vβ segments, and flat format files were put into Shannon software kindly provided by [21]. The resulting Shannon variability plots are found in figure 2.8. Alignments are provided in appendix C.

Shannon entropy (H) [22] is calculated by the formula:

$$H = -\Sigma\, p_i \log2\, p_i \quad \text{where } \Sigma \text{ represents the sum of } i \text{ from 1 to 20}$$

where p_i represents the proportion of amino acids of amino acid type i at a particular position in a sequence alignment. H ranges from 0 (invariant amino acid at that site) to 4.32 (equal representation of all 20 amino acids).

Many other studies use Kabat and Wu variability analysis to measure variability [23]. Kabat and Wu calculate variability at a site as the number of different amino acids found at that site divided by the relative frequency of the most common amino acid at that site. This method is extremely biased, showing the variability of only the most common one or two amino acids, over-accentuating both low and high diversity sites. Shannon entropy analysis gives a better estimate of the true amino acid variability at each site and is much less affected by small sample sizes or alignment or sequence errors [21].

2.1.6 Substitutions

Synonymous and non-synonymous substitutions were calculated when homologous segments were present in both species. In multi-member families the members that were more alike in the hypervariable regions were compared. The per residue substitution rate was calculated by dividing the number of substitutions in a region by the number of amino acids making up that region. CDRs are as defined by framework region depicted in figure 2.5. Where more than one nucleotide in a codon is changed, each nucleotide change is considered a substitution. Vβ1s2 and 3 were excluded from this analysis due to unclear homology. Vβ 19 includes a small frame shift and was excluded on this basis.

2.3 Results

Even though *Xenopus* species with 36 chromosomes, such as the LG hybrids, are *senu stricto* tetraploid compared to *Xenopus tropicalis*, they contain only one TCR locus per haploid genome. Each locus contains a single constant region [13] and the *gilli* and *laevis* constant regions can be differentiated. In mice and man, rearrangements at the TCR β locus occur mainly within one locus (*cis*) rather than between the two loci (*trans*) and this seems to be the case in *Xenopus* [24] [16]. Only 3 possible "hybrid" cDNAs which may correspond to *trans* recombination were recovered in over 450 sequences. We can therefore attribute each V and J sequence to either the *laevis* or the *gilli* locus by noting which constant region it is associated with. 9 new Vβ families have been found on the *laevis* locus thus bringing the total number of Vβ families to 19. 17 Vβ families were expressed from the *gilli* locus but hybridization experiments show the *gilli* locus contains all 19 families.

2.3.1 Cβ comparison

The *gilli* TCRβ constant region shows 95.7% identity with that of *laevis* differing by only 6 synonymous and 8 non-synonymous substitutions (figure 2.1). Despite this similarity, the *gilli*, unlike the *laevis* Cβ , possesses a site for N-linked glycosylation in the region just preceeding the a strand of the C1 domains Thus, the *laevis* Cβ and the axolotl Cβ1-3 are the only known Cβ segments which lack N-linked glycosylation [8]. The significance of this remains unknown. Another difference is the presence of F216 in the *gilli* sequence.

F216 in the f strand is thought to interact with aromatic residues at positions 152 and 153 to create a conformation necessary for CD3 δ and γ association, thus facilitating surface expression of the TCR complex [8, 25].

2.3.2 Identification of new Jβ segments

All of the Jβ segments described previously were found again in this screen [13]. In addition, 12 new Jβs were detected (figure 2.2). Jβs 2 ,3 ,8 ,11, and 22 are associated with both the *laevis* and *gilli* constant regions. Others, such as 7,10,13,14, and 20 differ by only a few base pairs and are grouped together on the assumption that they represent species specific alleles of the same Jβ segment. Where this assumption cannot easily be made, Jβs are assigned their own number awaiting clarification from genomic sequencing of the locus.

Using this nomenclature, the *laevis* and *gilli* loci contain 19 and 13 Jβ segments, respectively. Sterile transcripts containing RSS sequences were found for Jβs 2l, 2g, 3l, 9l, and 13l, thus defining the 5' end of these segments (figure 2.3). As expected, the J segments are well conserved in vertebrates, owing to the fact the J segment makes up the g strand of the immunoglobulin fold. The di-glycine bulge (GXG) is well conserved as well as T115 which has been shown to interact via Van der Waals forces and salt bridges with H156 in the constant region of crystallized murine TCRs [26]. This conservation suggests the intimate association of the Vβ and Jβ domains also occurs in *Xenopus* TCRβ chains.

The initial analysis of TCR sequences obtained by [13] suggested that Dβ2 sequence is GGGACTGGGGGGGC. Upon comparison of a greater number of sequences it is apparent that the Dβ2 sequence is most likely GGACTGGGGGAGC (figure 2.4).

2.3.3 Identification of additional Vβ segments

5' RACE was used to determine TCR segments present in LG3 and LG15 hybrids. 9 new Vβ families were identified along with the 10 previously described Vβs. Generally, *gilli* counterparts were found for *laevis* Vβs, and correspond to the equivalent of an allelic polymorphism in the LG hybrids (figure 2.5). Southern hybridization shows the TCR loci in LG3 and LG15 isogenic frog lines are the same (figure 2.6A). However, Vβ3 and 10 were only found to be expressed from the *laevis* locus. Hybridization of LG3 and 15 genomic

DNA with a Vβ3 probe indicates that 2 copies of the segment are present in the LG genome, but did not distinguish if each haplotype contributes a copy or if both bands were contributed by one haplotype. To clarify this, DNA from *Xenopus* haploid small egg tadpole was probed with a Vβ3 specific probe (figure 2.6B). Haploid LG3 tadpoles are produced when activated by UV irradiated sperm. These animals survive for several days, long enough to extract haploid DNA. Only one band was found in the haploid tadpoles corresponding to one of the two bands found in diploid animals. This shows that there is one BV3 segment on each locus. Therefore, each species contributes an allele of the Vβ3 segment, but the *gilli* allele is either a pseudogene or is very rarely used. Currently the first theory seems most likely because Vβ3 is quite well represented in both larval and adult populations (see chapter 5).

Several new family members have been found for Vβs 1, 6, and 8. Our initial hybridizations, carried out under high stringency conditions, did not allow for the hybridization of some of the more divergent members. Comparison of *Xenopus* TCR β sequences suggested that lower stringency conditions were unlikely to result in cross family hybridization (figure 2.6A). Our results are compiled in figure 2.7. Vβ1, 5, 6, 8,15 and 17 are multiple member families ([13] and figure 2.6A) and Vβs 1, 6, and 8 are present on both the *laevis* and *gilli* loci. There is some sequence evidence for possible additional Vβ1 and 8 segments as there are single instances of a Vβ8s3 like sequence associated with the *gilli* Cβ Vβ1s3g on figure 2.5) and a single instance of a possible fourth *laevis* associated Vβ1 sequence. Further sequence information will clarify the issue. Vβs 5, 15, and 17 show diploid expression at the mRNA level and additional Southern bands may represent rarely expressed family members or pseudogenes.

The *laevis* locus contains 25 Vβ segments, 24 of which are expressed. The *gilli* locus contains at least 25 segments, 20 of which rearrange and are transcribed. 17 and 19 Vβ families are used in *laevis* and *gilli*, respectively (figures 2.5 and 2.7). The new Vβ segments are readily recognizable as TCR segments maintaining conserved residues such as Q6, W34 and Y90, which are involved in interaction between the β sheets which make up the immunoglobulin fold, as are C23 and C92, which interact to form the intra-chain disulfide bond (figure 2.5) [27]). Y35, Q37 (exception is Vβ10 Q-H) and A92, residues involved in the interface with the alpha chain are also conserved. There are several residues that do not follow the conventional pattern of TCRβ chains. For instance, P8 is replaced in Vβs 3, 4, 6s1 and 6s2, 12 and 15 with a charged residue and in 7 and 8s2 with a H. The only other known TCRβ chain to have a charged residue at this position is the axolotl Vβ3 sequence (L29423). Another difference is the presence of a K residue at position 32 in *Xenopus* Vβs 2, 9 and 17 that could serve to lengthen the CDR1 and shorten the c strand. Only one *Raja* sequence, (U75756), shares this K. No known mammalian or avian sequences have a charged residue at this position. W65 residue is also unique amongst known TCRβ chains as is the R residue at position 79. Only *Xenopus* Vβs 3 and 15 and axolotl Vβ3 have a charged residue at this position. The relevance of these differences is not yet known, but the presence of unique charged residues within the strands which make up the β pleated sheets, such as R79, suggests these may be points of interaction with other strands of the Vβ or the TCRα chain and that these may differ from the crystallized mammalian structures.

TCR α and β V segments are known to be more similar to those of other species than to other segments within a species [8, 13]. Most Vβs show 30% or less aa similarity in intraspecies or interspecies comparisons. Vβs 13, 14 and 19 are most related to mammalian sequences. 13 shows 45% identity and 58% similarity to a goat sequence (U59410). 14 shows 43% identity and 59% similarity to human Vβ 20 (AAC80215) and *Xenopus* Vβ 19 shows 41% identity and 53% similarity to human Vβ 20 and murine Vβ 14 (AAC80215).

2.3.4 D-J-C transcripts

As previously reported, there are two functional Dβ segments in *Xenopus* [13]. Many D-J-C rearrangements are detected using our 5' RACE PCR technique. Jβs 2, 11 and 16 were commonly found in these rearrangements and always associated with the Dβ1 segment (figure 2.3). Similar transcripts are detected in mammals and Rainbow trout and their transcription thought to open the chromatin structure of the locus to permit to permit V to D-J-C rearrangement [28, 29]. Approximately half of the mRNA in human thymocytes consists of D-J joinings and such transcripts persist in mature cell lines and T cell hybrids [30].

2.4 Discussion

2.4.1 Why is there a single well-conserved Vβ segment?

It has been noted previously, that TCR Vβ sequences in poikilothermic vertebrates tend to be more similar between species than within species. A good example of this is the striking amino acid homology between the human Vβ 20 the murine Vβ 14 segments and *Xenopus* Vβ s 13,14 and 19, as well as shark Vβ 4, trout Vβ 1 and axolotl Vβ 5 sequences [8]. Because it is unusual to find such sequence similarity in TCR β variable regions between species it has been suggested that this one set of very similar Vβ segments may be derived from the same ancestral Vβ segment. It has also been argued that these "homologous" TCR Vβ segments may have co-evolved MHC molecules exhibiting minimal polymorphism or that they recognize a conserved antigen structure [12]. Current dogma states that CDRs 1 and 2 of both the TCR α and β chains are primarily responsible for interactions with the MHC molecule, leaving the more variable CDR3 to interact with the peptide [31]. Recent crystallography data suggests the TCR MHC/peptide interaction is more complex than first thought, in that all three CDRs, as well as the H4 region, can interact with the MHC, but do not always contact the same residues [32]. Direct co-evolution therefore seems unlikely. For similar reasons, it seems unlikely, as conserved as these segments are, that they can interact with the same ligands when complexed with different α chains and at varying temperatures. It is intriguing to note that mouse Vβ 14 and human Vβ 20 are the only Vβ segments found 3' of the second constant region and are transcribed in an orientation opposite to the rest of the locus [33, 34]. I propose that the conservation of this segment in multiple species is due to its placement in the locus rather than a specific function. In humans there are frequent null alleles of Vβ20 [34], arguing that this segment is not absolutely required. In *Xenopus*, the presence of 3 "homologous" Vβs 13, 14, and 19, may suggest a relatively ancient duplication in this region.

2.4.2 Patterns of divergence between *X. laevis* and *X. gilli* Vβ segments

Xenopus laevis and *Xenopus gilli* are closely related and thus a comparison of amino acid (aa) substitutions in the laevis and gilli alleles of the variable regions may provide insights into the evolutionary pressures on the TCR molecule [35, 36]. Taking into consideration only V regions present in both species, we find substitutions in the framework (FR) 1 and 3 regions account for 35% and 37% of total substitutions respectively, whereas the highly conserved FR2 region account for only 8% of the total. Even more striking is the observation that the CDR 1, 2, and H4 regions make up 5%, 4% and 7%, respectively, of the total substitutions. If we normalize this frequency of substitutions to the number of aas comprising a typical region, we find that in FR1 and 3, an average frequency of non-synonymous substitutions per residue of 6.9% and 5.3% respectively, whereas in FR2, the per residue frequency is only 3.5% per residue. The corresponding frequencies in the CDR1, 2 and H4 regions are 4.4%, 3.5% and 9.2% substitutions per residue. The relatively low frequencies of non-synonymous substitutions in the CDR1 and 2 regions are surprising considering the considerable variability observed between Vβ segment families within each species. Furthermore, the high substitution frequency seen in the H4 region suggests this region is under greater selective pressure to diversify than the other CDRs and marginally more than the framework regions. The rates of synonymous substitutions are higher than non-synonymous substitutions in both the CDR1 and CDR2, whereas, in the frameworks 2 and 3, the rates of both types of substitution are equally distributed in frameworks 2 and 3. Similar comparisons of orthologous TCRβ Vβ segments in primates show a higher rate of non-synonymous substitutions in CDR's 1 and 2 than in the framework regions [37-39]. This has been interpreted as evidence of purifying selection [39].

The TCR H4 region is known to interact with superantigens: proteins, usually produced by pathogens, which bind to the outside surface of the TCR and MHC class II molecules, thus cross-linking and activating up to 20% of peripheral T cells non-specifically [31]. Superantigens may have been an important selecting force for TCR segments in poikilothermic animals, which, like *Xenopus*, can get exposed to them very easily. If a tadpole can only produce a small finite number of peripheral T cells, superantigen activation of 20% of that small number of cells would likely represent a significant change in the animal's ability to protect itself from infection.

2.4.4 Measurements of TCR Vβ variability in several vertebrate species

The ability to bind a diverse range of peptide-MHC complexes requires variability in the CDRs of the TCR α and β chains. Many workers have stated that poikilothermic Vβ segments are as variable or more variable than mammalian segments [6, 8, 12] but no formal measurements have been used to date. Amino acid sequence variability can be calculated using the Shannon Entropy method [21]. Residues with entropies of 0 to 1 (H0) are generally structural or functional residues, whereas those between 1 an 2 represent functionally equivalent residues and often correspond to evidence of neutral drift. Shannon H2 sites (greater than 2) are highly variable sites [21, 40]. *Xenopus* Vβs possess 60–61 H2 sites as compared with 55, 39, 45 and 62 for axolotl, shark, human and mouse, respectively (figure 2.8) When one looks at H2.5 sites, *Xenopus* has 38-40 sites as compared with 17, 30, and 40 and 28 for shark, axolotl, mouse and human, respectively. Thus, overall, *Xenopus* and axolotl are as variable as mouse and human genomic Vβ repertoires. For shark and axolotl it is too soon to tell, for there was no exhaustive search for shark segments and there may be many more. The variability in the amphibian segments seems spread throughout the framework regions in addition to the CDRs. Mammalian CDR1s are based around the almost invariant Histidine29 at the center of the CDR1 loop table. This constitutes a significant drop in variability which is not seen in the poikilothermic plots. Recently, Al-Laziki et al, [2] have suggested that mouse and human CDRs 1 and 2 contain canonical structures and therefore take on specific conformations. Poikilotherms do not fall into the classification system of canonical CDR1 structures suggested by Al-Lazikani et al, [2].

The available shark segments lack the variability of the amphibians, a pattern also observed for the Ig light chain, where the Vs of cartilaginous fish always show less variability [40]. It has been suggested there is some limitation to the processes which generate variability in sharks. Alternatively, cartilaginous fish may rely on innate immunity and thus variability may not needed in the TCR [40]. These issues cannot be resolved until all of the TCR Vβ segments are cloned out of sharks.

2.4.5 How much can we infer from "homology"?

The diversity seen in the Vβs of *Xenopus*, and amongst poikilothermic vertebrates, remains intriguing. This could be due to longer evolutionary time to permit (neutral) divergence or could be a response to selection pressures. Our comparison of rate of synonymous and non-synonymous aa substitutions between the X. *laevis* and X. *gilli* Vβ segments suggests that the divergence is driven by selection pressures in *Xenopus*. Recognizing this variability and comparing it to that seen in mammals is a good first analysis, but if we hope to gain a deeper understanding how the T cell receptor functions in poikilotherms, we may not be able to use the analogy provided by mammalian crystal structures and functional studies. As more parts of the T cell receptor complex are cloned from non-mammalian vertebrates it will become possible to do functional studies on T cell function.

No text

Figure 2.1 Alignment of the Cβ regions of several vertebrate species.

Sequences were aligned using CLUSTAL and then manipulated. Dashes represent gaps and potential N-glycosylation sites are marked in yellow. Sites with over 80% identity are shaded in blue, and invariant residues are further marked with a black dot. Nearly invariant positions are marked with a red dot.

Figure 2.2 Alignment of *Xenopus* Jβ segments. *X. laevis* and *gilli* Jβ segments CDR3 regions from representative clones are shown. Spaces represent gaps. Putative D regions are boxed, and the longest representative J regions are shown in each case. Unattributed nucleotides have been left unboxed. To the right of the sequences the V, D, J and C segments for each clone are shown as well as the animal line (LG3 or LG15) from which the clone was isolated. L and G stand for *laevis* and *gilli*, respectively. Both the *laevis* and *gilli* "alleles" are given where unambiguous. Other sequences are only found in either *laevis* or *gilli*. Asterisks "*" mark out of frame rearrangements.

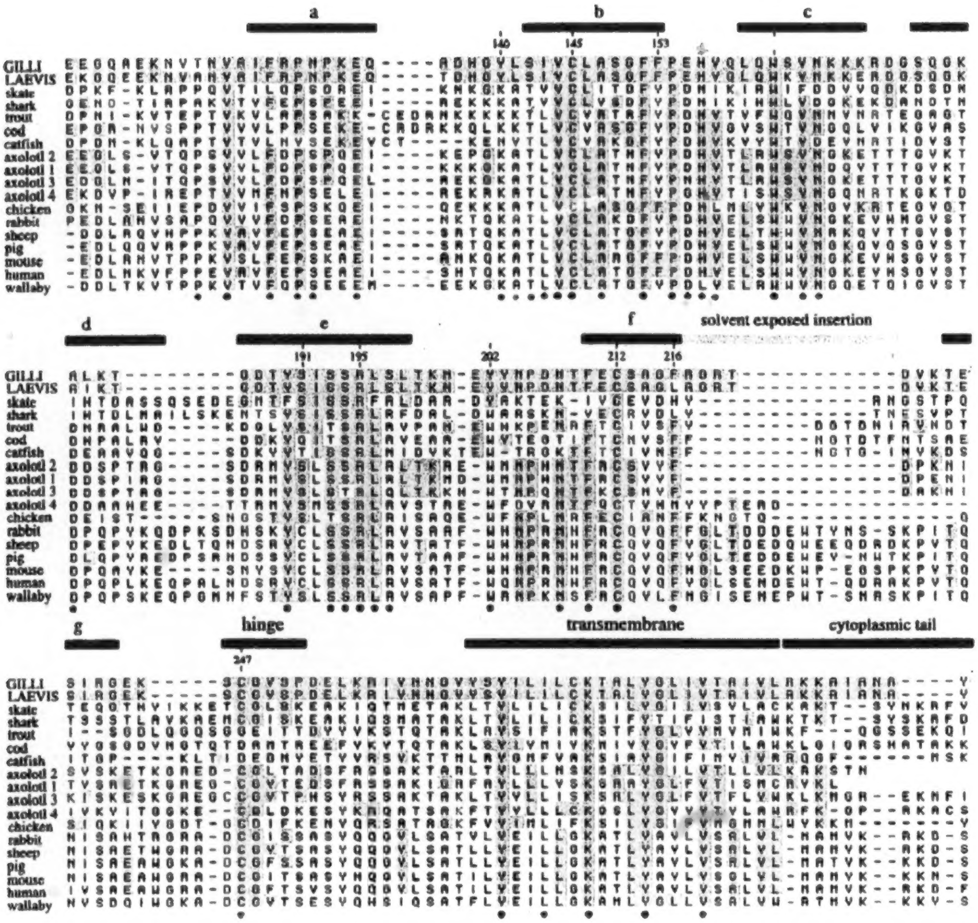

a
b
c
140
145
153
GILLI
LAEVIS
skate
shark
trout
cod
catfish
axolotl 2
axolotl 1
axolotl 3
axolotl 4
chicken
rabbit
sheep
pig
mouse
human
wallaby
d
e
f
solvent exposed insertion
191
195
202
212
216
g
hinge
transmembrane
cytoplasmic tail
247

Figure 2.2 Alignment of *Xenopus* Jβ segments. *X. laevis* and *gilli* Jβ segments

CDR3 regions from representative clones are shown. Spaces represent gaps. Putative D regions are boxed, and the longest representative J regions are shown in each case. Unattributed nucleotides have been left unboxed. To the right of the sequences the V, D, J and C segments for each clone are shown as well as the animal line from which the clone was isolated. L and G stand for *laevis* and *gilli,* respectively. Both the *laevis* and *gilli* "alleles" are given where unambiguous. Other sequences are only found in either *laevis* or *gilli*. Asterisks "*" mark out of frame rearrangements.

Xenopus TCR J beta sequences

Sequence	-----V--------		------D--------		-------------------------J-------------------------	V	D	J	C	HYBRID
T5D25L2	TGTGCTGCGAG	GYC	GGGACAGGGGG		CTGACAGACTGTATTTTGGCGACGGCACAATCCTCACTGTTCTT	5	1	1	L	LG15
	C A A R	X	G Q G A		D R L Y F G D G T I L T V L					
T8D24R1	TGTGCTGCGAGT		GACTGGGGG	ACTT	CTGGCAACAAGGTCACTTTTGGAGAAGGAACCATCCTCCATGTACTG	8	2	2	L	LG15
	C A A S		D W G	T	S G N K V T F G E G T I L H V L					
E99lan4	TGTGCCGCCAGTAA		CTGGGG		TACTTCTGCCAACAAGGTCACTTTTGGAGAAGGAACCATCCTCCATATACTG	4	2	2	G	LG3
	C A A S N		W G		T S A N K V T F G E G T I L H I L					
T3D78L2	TGTGCAGCGAGGA		CTGGGGGAG		CAAACACTGCTGCTTACTTTGGAGAAGGGACTGTGCTCACTGTGTTG	3	2	3	L	LG15
	C A A R		T G G		A N T A A Y F G E G T V L T V L					
T8D9L2	TGTGCTGCGAG	G	GACTGGGG		CACTGCTGCTTACTTTGGAGAAGGGACTGTGCTCACTGTGTTG	8	2	3	G	LG15
	C A A R		D W G		T A A Y F G E G T V L T V L					
T6D14-4	TGTGCATCTCAGAGTAT				TAATGACAGACAATATTTTGGAGAAGGCACAATCCTCACTGTTCTC	6.1	0	4	L	LG15
	C A S Q S I				N D R Q Y F G E G T I L T V L					
t9d9t1	TGTGCCAGCAG		GGAC	A	ATGCCGCTGTACAGTATTTTGGAGACGGCACAATCCTAACTGTTCTA	9	e	5	L	LG15
	C A S R		D		N A A V Q Y F G D G T I L T V L					
e77atr53	TGTGCGGCCAGTGAT	GC	GGACA	A	TTCTGGTTACGCACAGACGTTTGGAGATGGGACTCTTCTAACTGTGCTG	1.1	e	6	L	LG15
	C A A S D	A	D	N	S G Y A Q T F G D G T L L T V L					
5c31	TGTGCCAGCAGCA		CAGGGGGGG	CTAAC	GAGAGACAATATTTTGGAGACGGCACAATCCTTACTGTTCTG	9	1	7	L	LG3
	C A S S		T G G	A N	E R Q Y F G D G T I L T V L					
E91q3r12m	TGTGCTTCCAGCCA		G		AACGAGAGACAATATTTTGGAGACGGAACAATCCTCACTGTTCTG	15	e	7	G	LG3
	C A S S Q				N E R Q Y F G D G T I L T V L					
T2D23L1	TGTGCCAGCAGC		GGACA	T	AATTATCAAGCTTACTTCGGAGATGGAACCATTCTCACAGTTCTG	2	1	8	L	LG15
	C A S S		G H		N Y Q A Y F G D G T I L T V L					
3c32	TGTGCCAGCAGC		GAC		AATTATCAAGCTTACTTCGGAGATGGAACCATTCTCACAGTTCTG	9	e	8	G	LG3
	C A S S		D		N Y Q A Y F G D G T I L T V L					
T9D14L1	TGTGCCAGCAGC	AT	AGG	A	AATGCCGAGAGACAGTATTTTGGAGACGGCACAATGCTGGTTGTTTTA	9	1	9	L	LG7
	C A S S	I	G		N A E R Q Y F G D G T M L V V L					
6c19	TGTGCATCGAGTAT	GA	AGGGG		GCGGGCCAGAACAGACTGTACTTTGGAGATGGCACCATAGTCACAGTCCTA	6.2	1	10	L	LG15
	C A S S M		K G		A G Q N R L Y F G D G T I V T V L					
Ec5lan11	TGTGCCGGC	AGCCC	GGGACA		GGGCCAGAACAGACTGTATTTTGGAGATGGCACCATAGTCACAGTCCTA	18	e	10	G	LG15
	C A G	S P	G Q		G Q N R L Y F G D G T I V T V L					
t6d15T1	TGTGCATCTCAGAGTAT				ACATACCTACTCTGCAGCTTACTTTGGAGAAGGAACCATTGTCAGTGTTCTG	6.1	0	11	L	LG15
	C A S Q S I				H T Y S A A Y F G E G T I V S V L					
e60d621	TGTGCATCGAGCA		AGGGGGG		CTCTGCAGCTTACTTTGGAGAAGGAACCATTGTCAGTGTTCTG	6.2	e	11	G	LG15
	C A S S		K G G		S A A Y F G E G T I V S V L					
M9mc85	TGTGCCTGGAGCC		GGGACAG		TTCCTACAATGCTCAGTATTTCGGAGAGGGGACACTGCTCCATGTAATG	13	1	12	L	LG15
	C A W S		R D S		S Y N A Q Y F G E G T L L H V M					
e74atr28	TGTGCCAGCHGCACC		GACAGGGG	CA	CCCCACCAGCATTTGGGACTGGAACCTTCGTCAGCATCTTA	2	1	13	L	LG15
	C A S X T		D R G		T P P A F G T G T F V S I L					
T6D10L1	TGTGCATCTCAGAGTA		GG	GCTCTG	CCCCTCCAGTATTTGGGACTGGAACCTTCGTCAGCATCTTA	6.1	0	13	G	LG15
	C A S Q S		G	L C	P P V F G T G T F V S I L					
T3D9T1	TGTGCAGCGAGG		GACAG		TACTGGCCAGGCACAGTATTTTGGAGACGGCACAGTCCTGACTGTTCTA	3	1	14	L	LG15
	C A A R		D S		T G Q A Q Y F G D G T V L T V L					
Ec2a3t16			GGGGACAGGG		ATATGGCCAGACACAGTATTTTGGAGACGGCACAGTCCTAACTGTTCTA	0	1	14	G	LG3a *
			G Q G		Y G Q T Q Y F G D G T V L T V L					
T6D15T3	TGTGCATCTCAGAGT		GG		TTCTGGTGACAGGGTCTCTTTTGGAGAAGGGACCATCCTCCMTGTTCTG	6.1	e	15	L	LG15
	C A S Q S		G		S G D R V S F G E G T I L X V L					
C7q15t63	TGTGCTTGGAGTC	C	GACAG	CT	AGTGACAGAGCTCAGTACTTCGGAGAGGGCACTCGGCTGCTGGTTCTG	14	1	16	L	LG15
	C A W S	P	T	A	S D R A Q Y F G E G T R L L V L					
C11a3t41	TGTGCTTCCAGCCA	A	GACAGGG		AGGGCCAGACACAGTACTTTGGGGACGGCACAGTCCTAACTGTTCTA	15	1	17	G	LG3*
	C A S S Q		D R		G Q T Q Y F G D G T V L T V L					
12c19	TGTGCATCTCAGAGTA		AGGGGG	CCGT	CGCTGACAGACTGTATTTTGGTGATGGCACAATCCTCACTGTTCTG	6.1	1	18	G	LG15
	C A S Q S		K G	A V	A D R L Y F G D G T I L T V L					
E7mc75	TGTGCGGCAGTGATGC	G	GGGGACAGGGG		TACCGAGAGACAATATTTTGGAGAGGGCACAATCCTCACTGTTTTG	1.1	1	19	G	LG15
	C A A S D	A	G D G		T E R Q Y F G E G T I L T V L					
Ec4lan7					TGAGAgACAATATTTTGGAGACGGCACAATCCTCACTGTTCTG	0	0	20	L	LG3
					E R Q Y F G D G T I L T V L					
Ec4lan4	TGTGCTTGGAGTC	A	GACAGGGAGG		AATGACAGACAATATTTTGGAGACGGCACAATCCTCACTGTTCTG	14	1	20	G	LG3
	C A W S	Q	T G R		N D R Q Y F G D G T I L T V L					
ABI 25	TGTGCGGCCAG		GAC	GCGT	ACCGCTGACAGACTGTATTTTGGCGACGGCACAATCCTCACTGTTCTC	1.1	e	21	L	LG15
	C A A R		T	R	T A D R L Y F G D G T I L T V L					
t1d23T1	TGTGCGGCCAGTGA		CA		ATGCCGCTGTACAGTATTTTGGAGATGGCACAATCCTAACTGTTCTA	1.1	e	22	L	LG15
	C A A S D				N A A V Q Y F G D G T I L T V L					
C7q15t64	TGTACCAGCAGCACCG		GACA	T	AATGCCGCTGTACAGTATTTTGGAGATGGCACAATCCTAACTGTTCTA	17	e	22	G	LG15
	C T S S T		G H		N A A V Q Y F G D G T I L T V L					

```
A.DB1.nonamer.heptamer.sequences                                                                                      J  C HYBRID
M9MC75    CAATTCTTTTTGTATGAGAGTGTAACGTTGTGGGGACAGGG       TTCTGGCAACAAGGTCACTTTTGGAGAAGGAACCATCCTCCATGTACTG  2  L LG15

c45sam10  CAATTCTTTTTGTATGAGAGTGTAAGGTTGTGGGGACAGGGGGGGC    CTGGCAACAAGGTCACTTTTGGAGAAGGAACCATCCTCCATGTaCTG  2  L LG15
4c18      CAATTCTTTTTGTATGAGAGTGTAACTGTGTGGGGACAGGG                 CTGCTGCTTACTTTGGAGAAGGGACTGTGCTCACTGTGTTG  3  L LG3
C46sam14  CAATTCTTTTTGTATGAGAGTGTAACGTTGTGGGGACAGGG               CACTGCTGCTTACTTTGGAGAAGGGACTGTGCTCACTGTGTTG  3  L LG15
17-N20    CAATTCTTTTTGTATGAGAGTGTAACGTTGTGGGGACA          TAATGCCGCTGTACAGTATTTTGGAGACGGCACAATCCTAACTGTTCTA  5  L LG
c10a3t44  CAATTCTTTTTGTATGAGAGTGTAACTGTGTGGGGACA         CTTCTGGTTACGCACAGACGTTTGGAGATGGGACTCTTCTAACTGTGCTG  6  L LG3
C9q15t58  CAATTCTTTTTGTATGAGAGTGTAACKTtGTGGGGACA       GAATTCTGGTTACGCACAGACGTTTGGAGATGGGACTCTTCTAACTGTGCTG  6  L LG15
8c17      CAATTCTTTTtGTATGAGAGTGTAACGTTGTGGGGACAGGGGG           ATTATCAAGCTTACTTCGGAGATGGAACCATTCTCACAGTTCTG  8  L LG15
20-N23    CAATTCTTTTTGTATGAAAGTGTAACGTTGTGGGGACAGGGGGGG        CCTTATCAAGCTTACTTCGGAGATGGAACCATTCTCACAGTTCTG  8  L LG
21-N24    CNATTCTTTTTGTATGAGAGTGTAACGTTGTGGGGACAGGGGGG          TTATCAAGCTTACTTCGGAGATGGAACCATTCTCACAGTTCTG  8  L LG
14-N16    CAATTCTTTTTGTATGAGAGTGTAACGTTGTGGGGACA                CTCTGCAGCTTACTTTGGAGAAGGAACCATTGTCAGTGTTCTG  11 L LG
E84qtr75  CAATTCTTTTTGTATGAGAGTGTAACTATGTGGGGACA            CCTACTCTTCAGCTTACTTTGGAGAAGGAACCATTGTCAGTGTTCTG  11 L LG15
C333      CAATTCTTTTTGTATGAGAGTGTAACgNTGTGGGGACAGGGGGGG    CCATGCTCTGCAGCTTACTTTGGAGAAGGAACCATTGTCAGTGTTCTG  11 L LG15
Ec2a3t16  CAATTCTTTTTGgATGAGAGTGTAACGTTGTGGGGACAGGG       ATATGGCCAGACACAGTATTTTGGAGACGGCACAGTCCTAACTGTTCTA  14 G LG3
Ec4lan9   CAATTCTTTTTGTATGAGAGTGTAACKTTGTGGGGACAG                   AGCTCAGTACTTCGGAGAGGGCACTCGGCTGCTGGTTCTG  16 L LG3

B.                                                                                                                    J  C HYBRID
Ec2a3t7             CCCTTTTTGTGCCTCTTTCTTGCCCTGTGTCCTCTCTCTGGCAACAAGGTCACTTTTGGAGAAGGAACCATCCTCCATGTACTG  2  L LG3
8c18          GGGGGAGTTTTTATGCGCCCATGTCGGGCTGTGACTTCTGGCAACAAGGTCACTTTTGGAGAAGGAACCATCCTCCATGTACTG         2  G LG15
EM44          ACGGGGCTTTTTGTACAATAATGTAACAATGTGGGACTTTACACTGCTGCTTACTTTGGAGAAGGGACTGTGCTCACTGTGTTG         3  L LG15
C9q15t60      GGACAGGATTTAGAAGGGGTAAATGTGACTGTGAATGCCGAGAGACAGTATTTTGGAGACGGCACAATGCTGGTTGTTTTA            9  L LG15
c81s3         AGTGGATTCAATTG[illegible]GGTGCTGGTGTCACTGTGAGCTCTGCCCCACCAGCATTTGGGACTGGAACCTTCGTCAGCATCTTA  13 L LG15
```

Figure 2.3 D-J-C or J-C transcripts are common

A Representative D-J rearrangements. The heptamer/nonamer sequences for D1-Jβ sequences are underlined. Spaces represent gaps. To the right of the sequences the J and C segments are given. B. The heptamer/nonamer sequences for five Jβ segments are underlined.

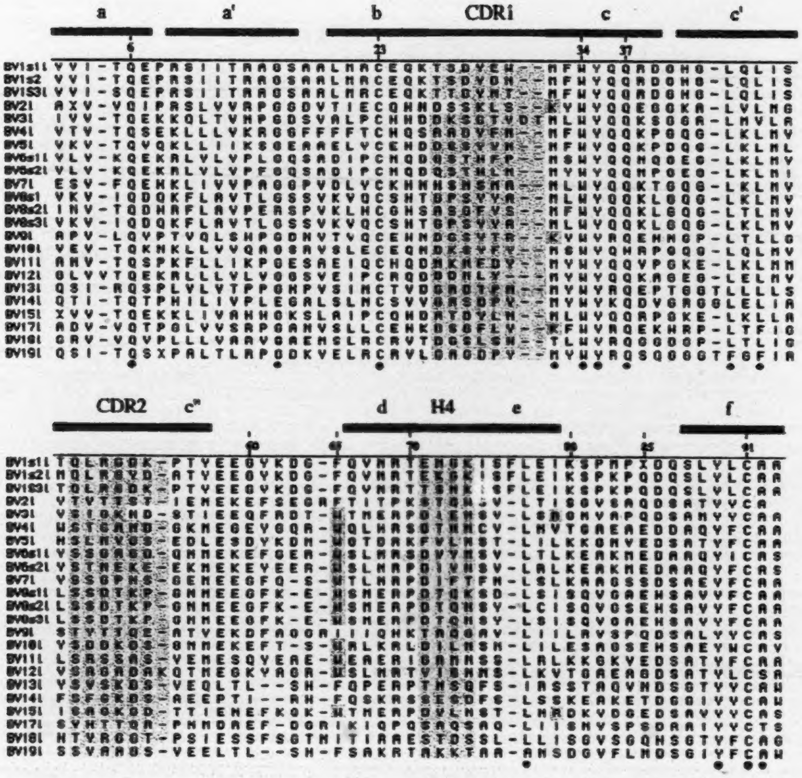
```
MOUSE                   GGGACTGGGGGGGC

RAT                     GGGACTGGGGGGGC

HUMAN                   GGGACTAGCGGGAGGG

XCONSENUS DB2              GGACTGGGGGAGC
....................................................................................... ......V....D....J

T102T7  ACTGGTGCGCTGTCA   GACTGGGG              ACACTGCTGCTTACTTTGGAGAAGGGACTGTGCTCACTGTGTTG 10    DB2  3

T1D15-1 CGGCCAG           GACTGGGG            AAACACTGCTGCTTACTTTGGAGAAGGGACTGTGCTCACTGTGTTG  1    DB2  3

B3      GCGAGGAAAT       GGACTGGGGGAG        AAAACACTGCTGCTTACTTTGGAGAAGGGACTGTGCTCACTGTGTTG  3    DB2  3

T6D15-7 GAGT               ACTGGGGGAGC            TGCTGCTTACTTTGGAGAAGGGACTGTGCTCACTGTGTTG  6    DB2  3

T2D9T2  GTGCCAGCAGC        ACTGGGGGAGC            TGCTGCTTACTTTGGAGAAGGGACTGTGCTCACTGTGTTG  2    DB2  3

T6D9-2  CGAGTATAT          ACTGGGGA              CTGCTGCTTACTTTGGAGAAGGGACTGTGCTCACTGTGTTG  6    DB2  3

T8D9N2L TGCTGCGA        GGGACTGGGG             CACTGCTGCTTACTTTGGAGAAGGGACTGTGCTCACTGTGTTG  8    DB2  3

T3D78L2 GTGCAGCGA        GGACTGGGGGAGC        AAACACTGCTGCTTACTTTGGAGAAGGGACTGTGCTCACTGTGTTG  3    DB2  3

B20j    GCGAGTGA             TGGGGG           TGGTTACGCACAGACGTTTGGAGATGGGACTCTTCTAACTGTCCTG  8    DB2  6

B12     GCCAGCCT             TGGGGGAGCC              GCTGCTTACTTCGGACATGGAACCATTCTCACAGTTCTG  4    DB2  8

JOHN68    TGCGAGT         gACTGGgGG      ACTTCTGGCAACAAGGTCACTTTTGGAGAAGGAACCATCCTCCATGTACTG  ?    DB2  2

P15Q15T  CTGGAGTCT       GGACTGGGGA             ACACTGCTGCTTACTTTGGAGAAGGGACTGTGCTCACTGTGTTG 13    DB2  3

E81QTR17  GCCAGTGA           TGGGGGAGC        TGGCAACAAGGTCACTTTTGGAGAAGGAACCATCCTCCATGTACTG 1s3   DB2  2

E14MC75   TCAGCGA       GGACTGGGGGAGC       TAACACTGCTGCTTACTTTGGAGAAGGGACTGTGCTCACTGTGTTG 12    DB2  3
```

Figure 2.4 Comparison of mammalian and *Xenopus* Dβ2 segments.

A consensus *Xenopus* Dβ2 sequence is apparent from alignment of CDR3 regions. This consensus differs slightly from the previously published sequence reported in Chretien et al, [13] and in Charlemagne et al, [8]. In each case, the clone name is shown to the left of each sequence. The 3' end of the Vβ segment is shown in each case followed by an aligned Dβ sequence. J beta sequences are aligned to the right of each Dβ. The V, D and J segment used in each sequence is given to the right of each sequence.

Figure 2.5 Alignment of *Xenopus* Vβ segments found to date

Alignments of the predicted amino acid sequences of variable regions associated with the A. *laevis* constant region and B. the *gilli* constant region are shown. Sequences are numbered according to Al-Lazikani et al.[2] Sequences have been grouped into families based upon 75% DNA homology and phylogenetic analysis (data not shown). Alignments were produced in Lasergene using a PAM 100 matrix and then modified by hand. The strand structure of the V domain is shown, and hypervariable regions are highlighted in blue. Putative sites for N-linked glycosylation are shown in yellow. Residues in green are unique to *Xenopus*. Invariant positions are marked with a black dot, and nearly invariant positions with a red dot. Dashes represent gaps.


a
a'
b
CDR1
c
c'
6
23
34
37
BV1s1l
BV1s2
BV1S3l
BV2l
BV3l
BV4l
BV5l
BV6s1l
BV6s2l
BV7l
BV8s1
BV8s2l
BV8s3l
BV9l
BV10l
BV11l
BV12l
BV13l
BV14l
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H4
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f
60
67
70
80
85
91

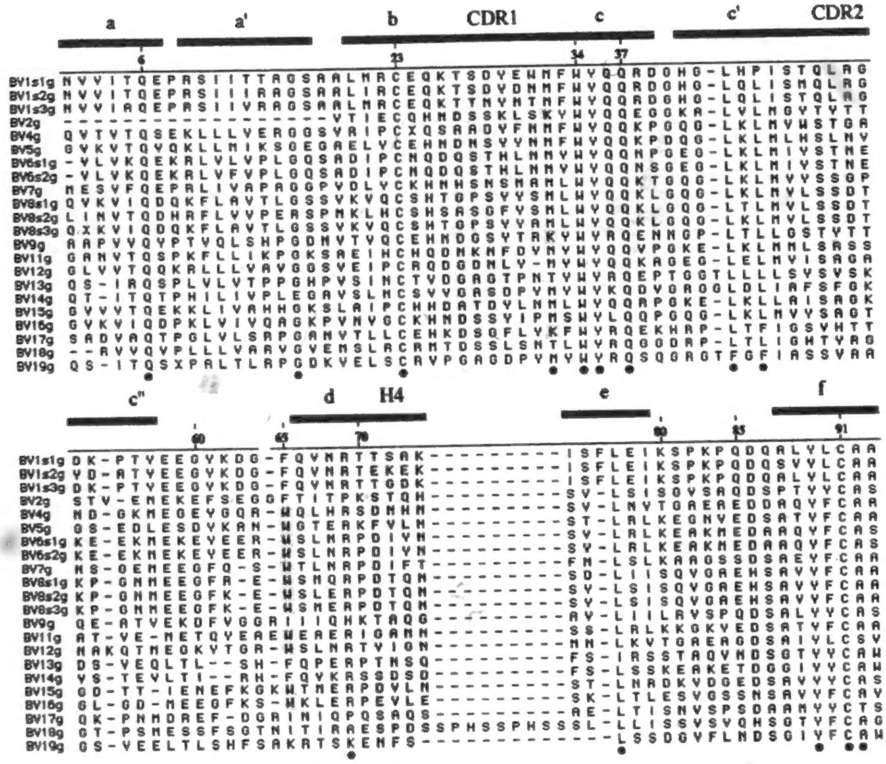
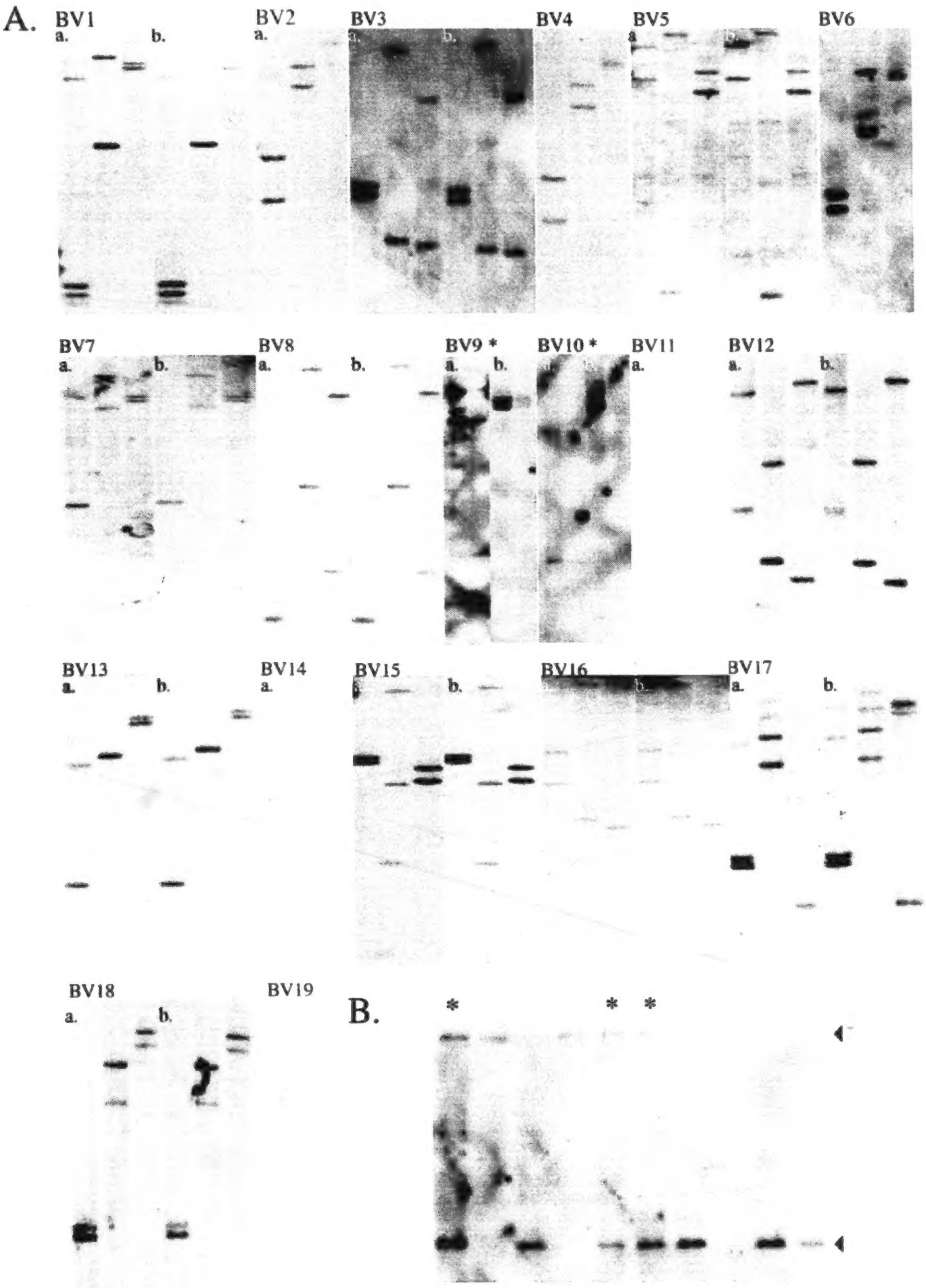

a
a'
b
CDR1
c
c'
CDR2
6
23
34
37
BV1s1g
BV1s2g
BV1s3g
BV2g
BV4g
BV5g
BV6s1g
BV6s2g
BV7g
BV8s1g
BV8s2g
BV8s3g
BV9g
BV11g
BV12g
BV13g
BV14g
BV15g
BV16g
BV17g
BV18g
BV19g
c''
d
H4
e
f
60
65
70
80
85
91

Figure 2.6 Southern tranfer analysis of the number of Vβ segments in *Xenopus*

A. Genomic DNA from the (a) LG15 or (b) LG3 hybrid frog line, was digested with Pst I, EcoRI or HindIII. In each case lanes were loaded in the order, Pst I, EcoRI or HindIII, from left to right. Southern transfers were hybridized with the indicated V domain specific probes and washed at low stringency. Asterisks (in A) indicate filters where the DNA was digested with BamHI and HindIII and loaded from left to right. These results confirm that LG3 and LG15 have the same TCR locus as expected by previous Southern results in Chretien et al. [13]. Note that photos have been rescaled for this figure and therefore the spacing of bands is not uniform. B. Vβ3 is present in both the *laevis* and the *gilli* loci. DNA from small egg progeny was digested with NcoI. Small egg progeny are haploid individuals and thus show the contribution of either the *laevis* or the *gilli* loci from the LG15 mother. The Southern transfer was hybridized with Vβ3 probe. Asterisks "*" indicate diploid individuals, whereas the remainder are haploid.


A.
BV1
a.
b.
BV2
a.
BV3
a.
b.
BV4
BV5
a
b
BV6
BV7
a.
b.
BV8
a.
b.
BV9 *
a.
b.
BV10 *
BV11
a.
BV12
a.
b.
BV13
a.
b.
BV14
a.
BV15
b.
BV16
b.
BV17
a.
b.
BV18
a.
b.
BV19
B.
*
*
*

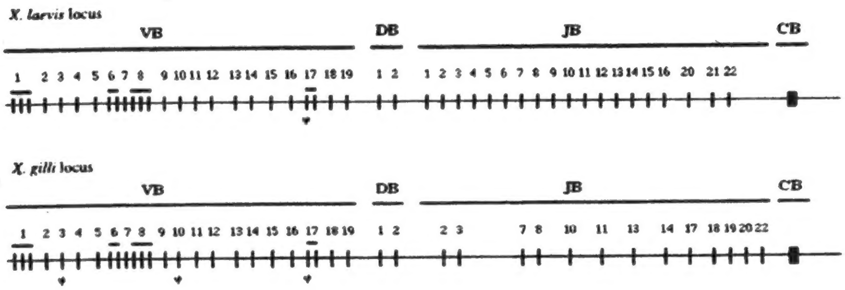
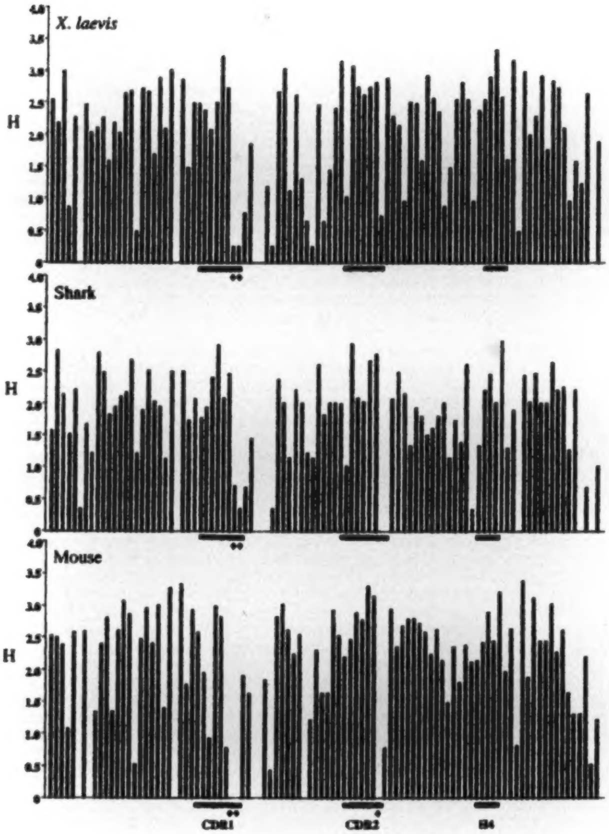

X. laevis locus
VB
DB
JB
CB
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
1 2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 20 21 22
X. gilli locus
VB
DB
JB
CB
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
1 2
2 3 7 8 10 11 13 14 17 18 19 20 22

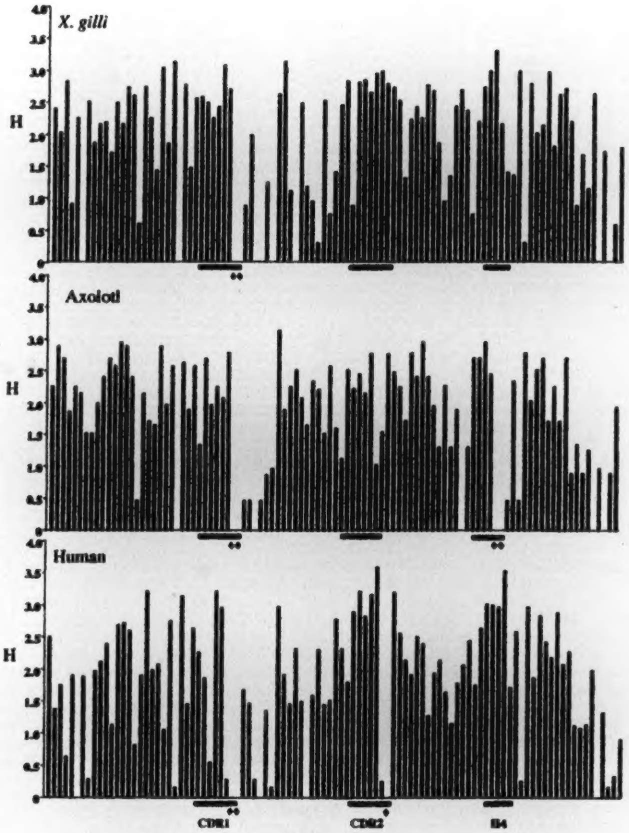
Figure 2.7 Schematic diagram of the *laevis* and *gilli* TCR β loci

Segments consistently associated with either the *laevis* or *gilli* Cβ are shown. Note that segments were named in their order of isolation and that the order or organization of the segments in each locus is not known. Multiple member Vβ families, as shown by analysis of the Southern data, are indicated by horizontal bars. Pseudogenes (φ) are indicated where no corresponding sequences have been isolated from cDNA libraries.

Figure 2.8 Shannon entropy variability plots

Sequence alignments of the Vβs in *Xenopus laevis, Xenopus gilli*, shark, axolotl, mouse and human were performed. Alignments were then entered into the Shannon software kindly provided by [21]. and results plotted. Positions are equivalent in the six panels. The location of the hypervariable loops is shown. See text for a discussion of Shannon entropy values. Alignments are shown in Appendix C.


X. laevis
Shark
Mouse
H
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
CDR1
CDR2
H4


X. gilli
Axolotl
Human
H
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
CDR1
CDR2
H4

No Text

References

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No Text

Chapter 3

3.0 Characterization of a polyclonal serum recognizing *Xenopus* CD3ε

No Text

3.1 Introduction

After looking at the level of diversity in the genomic repertoire we wanted to study the actual TCR diversity used in tadpole and adult *Xenopus*. In tadpoles, it was not actually known exactly when T cells began to leave the thymus and enter the peripheral tissues of the animal. Also, because what little was known was based upon antibody staining and functional assays it was not clear if all T cell populations could be assayed by these methods. A new reagent was tested by a collaborator, Thomas Göbel. This polyclonal antiserum was raised against a highly conserved epitope in the cytoplasmic tail of CD3ε, a component of the TCR associated signaling complex. This serum shows specific staining of CD3ε on all tested mammals and also chickens. Thomas Göbel showed that this reagent can coimmunoprecipitate the *Xenopus* TCR complex [1].

Here I present work that shows this reagent can specifically stain *Xenopus* T cells allowing us to visualize T cell subsets which were previously unknown in *Xenopus*. The advantages and limitations of the use of this reagent are discussed.

3.1.1 The CD3 complex

The CD3 complex is well characterized in mammals and in chicken where it is necessary for signaling through the TCR [2]. The complex consists of CD3ε, γ, δ and ζ chains in mammals and CD3 ε, γ/δ and ζ chains in chicken [2-4]. In other vertebrates, information concerning this complex has been limited to the cloning of the genes for TCR and CD3 chains from different species because no immunoprecipitation was possible due to an unavailability of immunological reagents. TCR chains from different vertebrate classes show a low overall homology, with conservation of mainly important structural residues [5].

3.1.1.1 Non-mammalian CD3 homologues

Non-mammalian CD3 homologues have been identified in chickens, frogs, newts, flounder (a teleost fish) and the sterlet (a chondrostean fish) [3, 6, 7]. The function of the CD3 complex seems to be conserved in chicken. In fact, the structure of the chicken

CD3ζ chain can fully rescue the function of the mouse ζ-ζ homodimer in a ζ deficient cell line [8]. The chicken, *Xenopus laevis*, and Iberian ribbed newt (*Pleurodeles waltl*) (AF397406) homologues of the CD3 γ/δ gene are likely to represent an ancestral form of the recently duplicated mammalian CD3γ and CD3δ genes. CD3ε homologues have been cloned from chickens, flounder (a teleost fish) and the sterlet [1, 3, 6, 7]. The extracellular domains of these homologues are very divergent, showing a conservation of only residues involved in the interchain disulfide bond and a CXXC motif in the membrane proximal domain [9, 10]. The intracellular domain however, contains a highly conserved proline rich domain (figure 3.1).

3.1.2 A scarcity of reagents has severely limited the study of many aspects of non-mammalian immunity

Despite the functional conservation of parts of the T cell receptor complex, the sequences are seldom conserved enough to allow antibodies to cross-react between species. The characterization of *Xenopus* T cells has long been complicated by this unavailability of specific antibodies limiting work to CD5 (2B1), a general T cell marker [11]; XT-1, a marker of a T cell subset [12]; CTX, a cortical thymocyte marker [13]; and two anti-CD8 antibodies which recognize the CD8α and CD8β chains respectively [14, 15]. None of these markers is certain to recognize *Xenopus* γδ T cells.

3.1.3 A commercially available antiserum which recognizes CD3ε in many species

Two antibodies raised to the highly conserved proline rich cytoplasmic domain of human CD3ε are exceptional in that they recognize CD3ε in all tested mammalian species, as well as chickens, ducks, and *Xenopus* [10, 16]). This antiserum has recently been used to characterize the make-up of the *Xenopus* TCR complex. Co-immunoprecipitation of *Xenopus* TCR, with the anti-CD3ε polyclonal serum, yields four bands of 110, 75, 18 and 17 kDa under non-reducing conditions and three of 40, 35 and 18 kDa after reduction. The 75 kDa protein is composed of a 40 kDa, acidic TCR α chain and a 35 kDa, basic TCR β chain [1]. Associated are two proteins of 19 kDa which, after deglycosylation resolve into 19 and 16.5 kDa proteins representing the CD3ε and CD3 γ/δ chains respectively. Thymocytes, splenocytes and the *Xenopus* B3B7 thymoma cell line express the TCR complex on their surface [1].

Here I extend these finding by showing that the polyclonal antiserum can play a useful role in the identification and localization of previously uncharacterized T and perhaps NK cell populations in *Xenopus*.

3.2 Material and methods

3.2.1 Animals

All animals were maintained at the Basel Institute for Immunology animal colony. LG15 animals were produced by gynogenesis as previously described [17]. Outbred *Xenopus laevis* animals were used for the cell sorting experiments.

3.2.2 Antibodies

The CD3ε polyclonal antiserum produced against a synthetic peptide derived from the intracellular domain of human CD3ε (see figure 3.1) and purified on human CD3ε was obtained from DAKO (Heidelberg, Germany). A monoclonal antibody for the same epitope is available from Serotec (Oxford, UK).

3.2.3 Cell lines

The B3B7 *Xenopus laevis* thymoma line was maintained in amphibian SF medium at 27°C as previously described [18].

3.2.4 Immunohistology

Whole tadpoles, adult tissues and tumors were frozen in O.C.T. embedding medium (Tissue Tek), cryosectioned (6μ), fixed 1 minute in acetone, and blocked with 1.5% BSA in PBS. The CD3ε antiserum (1:25 dilution) was absorbed twice, volume to volume on *Xenopus* red blood cells (XRBC). The absorbed serum was further diluted 1 to 100 and incubated with the sections for 2 hours at room temperature. Sections were washed 3 times in PBS and again blocked before a 30-60 minute incubation with FITC or

TR labeled goat-anti-rabbit Ig (SBA, Birmingham, AL) antibody (1:200 dilution) (Southern Biotech). Anti-CD8 staining was performed with the AM22 mAb [14](1:75 dilution) followed by a fluorochrome conjugate of goat-anti-mouse-IgM (SBA).

3.2.5 Cell preparations

Single cell suspensions of *Xenopus laevis* splenocytes were obtained using standard procedures. For sorting, cells were stained with AM22 and 10A9 [19] mAbs (anti-IgM) for 2 hours on ice, followed by *Xenopus laevis* red blood cell adsorbed PE conjugated anti-mouse IgM (1:200) and FITC conjugated anti-mouse IgG1 (1:200). Cell sorting was performed on a FACs Vantage and sorted cells used for cytocentrifuge preparations.

^{35}S Labeling of the B3B7 thymoma

Log phase B3B7 cells [20] were collected and pelleted (1000 rpm 12min), rinsed twice with RT or 27°C amphibian PBS (APBS), counted and scored for a minimum 80% viability. Cells were resuspended at ~2.5 x 10^7 cells/mL in pre-warmed (27°C) labeling media (Cell culture Water (Sigma) 785 μl, L-Glutamine 65 μl, dialyzed FCS 650 μl, and Cys/Met/L-Glut free Dulbecco's Modified Eagle's Medium (Sigma) 5 ml). 200 μl of ^{35}S Cys/Met (7.15mCi/ 500μl, Amersham) was added per 2 ml labeling media. Cells were labeled 2 hours at 27.0°C with gentle agitation every 0.5 hours. Cells were subjected to centrifugation at 1000 rpm at 4°C and the supernatant was removed. Finally, cells were rinsed 5x with 12 mL of 4°C APBS. This protocol and advice was provided courtesy of Jacques Robert and Nick Cohen.

3.2.7 Digitonin lysis of B3B7

Digitonin lysis buffer (1% digitonin (calculated on recrystallized digitonin), 0.3M NaCl, 0.1M Tris pH 7.4, 0.01M EDTA, 0.2% NaN_3, Iodoacetamide 0.04M) was used in all cases to maintain the integrity of the complex. One tablet of Complete™ protease inhibitor (Roche Diagnostics) per 25 mL of lysis buffer was added just before lysis. Lysis was carried out at 1X 10^8 cells/mL for one hour on ice, and then centrifuged at 15 000g for

30 minutes. The supernatant was then removed and kept at 4°C. This protocol and advice was provided courtesy of Thomas Göbel.

3.2.8 Immunoprecipitation of B3B7

Antibodies were conjugated to plastic ELISA plates (see Appendix A). Lysates were incubated ON at 4°C with gentle agitation, and then centrafuged after which the supernatant was removed. The wells were then washed 10x with lysis buffer containing 0.1% digitonin. After last wash, Laemmli buffer was added to the well and incubated at 55°C for 25 min, followed by centrifugation. The supernatant was then removed and loaded onto a NuPage precast SDS-PAGE gel.

3.2.9 Electrophoresis

Proteins were resuspended in Laemmli buffer and then separated on a gel gradient of 4 to 20% polyacrylamide under non-reducing conditions. Gels were fixed, treated with an enhancer (Enlighten), dried and exposed to Kodak BIOMAX MR film at RT for several days.

3.2.10 Results

3.2.11 Comparison of the available polyclonal antiserum and monoclonal antibody

Two reagents to the conserved proline rich region are available. The first is a rat anti-CD3ε peptide monoclonal antibody. The second is a rabbit anti-human CD3ε polyclonal antiserum. This serum was purified by DAKO on human CD3ε. To avoid non-specific background staining in these studies, the polyclonal serum was extensively adsorbed on *Xenopus* red blood cells.

3.3.2 Immunoprecipitation of ^{125}I labeled B3B7

Immunoprecipitation of ^{125}I labeled B3B7 tumour cells using both the monoclonal and polyclonal antibodies isolated proteins of a size consistent with the TCR $\alpha\beta$ complex (figure 3.2)

3.3.3 Immunoprecipitation of lysates of ^{35}S labeled B3B7 cells is less straightforward

Surface labeling using radioiodination (figure 3.2) or biotinylation (data not shown) showed only the TCRβ complex suggesting that this serum could be useful in purifying components of the *Xenopus* TCR complex for protein sequencing and subsequent cloning.

However, immunoprecipitation of lysates from ^{35}S labeled cells showed that many intracellular proteins were co-immunoprecipitated. In this respect the monoclonal and polyclonal antibodies for CD3ε gave equivalent results. This may have been due to cross-reaction with an epitope shared by many intracellular proteins (figure 3.3). However, a similar pattern is seen for the XT-1 mAb, which has previously been shown to immunoprecipitate a single surface 120 kDa molecule on *Xenopus* T cells after radiodination [14]. However, under these conditions, in a lysis buffer which favours the integrity of complexes, XT-1 can immunoprecipitate a number of intracellular proteins as well (figure 3.3 lane H). This greatly complicated the problem of protein isolation and sequencing and all attempts to isolate parts of the TCR complex cleanly, failed.

3.3.4 Fluorescence activated cell sorting (FACs)

This reagent is specific for an intracellular epitope thus limiting its use in cell sorting. Several attempts to permeablize cells with saponin and stain with this reagent failed to stain cells specifically.

3.3.5 Tissue staining

The monoclonal and polyclonal sera differed in their staining of tissues. The absorbed pAb was much more specific than the mAb for still unknown reasons (data not shown). The duration of the staining time with the pAb was limited to a few hours at most, for overnight incubations consistently resulted in mild to overwhelming background staining (data not shown). Because of this difference in tissue staining all further experiments employed the absorbed polyclonal antisera.

3.3.5.1 Sorted *Xenopus laevis* T lymphocytes are specifically detected with the CD3ε antiserum

Because of the ambiguous results obtained in immunoprecipitation and the need to limit the staining time of cells, we wanted to determine if known T cell populations could be stained with the CD3ε antiserum. For this we stained *Xenopus* splenocytes with antibodies to CD8 and Ig as T and B specific markers, and sorted them using a fluorescent activated cell sorting machine. (We would like to thank Mark Dessing and Tracy Hayden for cell sorting.) Because the CD3ε pAb recognized an intracellular epitope it was not possible to stain cells directly for CD3ε.

Splenocytes were sorted into CD8$^-$ Ig$^-$, CD8$^+$ Ig$^-$, and Ig$^+$ populations (figure 3.4) mounted on slides and tested for CD3ε immunohistological staining. Appropriate controls using only secondary reagents were negative and used to define autofluorescence and set gates (data not shown). A subpopulation of the CD8$^-$ Ig$^-$ population reacted with the CD3ε antiserum (figure 3.5a). These cells most likely represent CD4$^+$ T cells, whereas Ig$^+$ cells were negative for CD3ε staining (data not shown). All CD8$^+$ cells expressed CD3ε at varying intensities (figure 3.5b). Unsorted splenocytes also stained with varying intensities (figure 3.5c and figure 3.6). The *Xenopus laevis* B3B7 thymoma cell line [20] is also stained by the CD3ε antiserum (figure 3.5d).

3.3.5.2 T cells in the adult gut are CD3ε

Staining of the intestine with an anti-CD8 mAb (AM22) and the CD3ε antiserum revealed two major populations, CD3ε$^+$ CD8$^+$ cells and single CD3ε$^+$ cells that did not express CD8 (figure 3.7). These cells were found both in smooth muscle (figure 3.7 a, b) and in gut associated lymphoid tissue (GALT) aggregations (figure 3.7 i, j, k, and l). More than two populations of cells seem to exist in the gut. CD3ε$^+$ cells that do not express CD8 may represent either CD4$^+$ lymphocytes or intestinal NK cells expressing CD3ε.

Similar results were obtained on splenic sections where both subpopulations were clearly observed (figure 3.6). As expected, most $CD8^+$ cells appear to coexpress $CD3\varepsilon$ to varying degrees. In addition to $CD3\varepsilon^+$ $CD8^+$ and single $CD3\varepsilon^+$ cells, there are $CD8^{hi}CD3\varepsilon^{lo}$cells found in small clusters (figure 3.7d). It is currently unclear what type of of cells these represent.

3.3.5.3 $CD3\varepsilon^+$ cells are found in tadpole tissues

On whole tadpole sections, $CD3\varepsilon$ positive cells were detected from day 5 post fertilization on and were observed in the thymus, liver, spleen, esophagus, skin, stomach and in the lining of the gut (figure. 3.8 and see chapter 4). Thus, the $CD3\varepsilon$ antiserum is an important tool to visualize non-mammalian T cells and is used to analyze the tissue distribution and ontogeny of *Xenopus laevis* T cells, in chapter 4.

3.3.6 TCR β rearrangements in the B3B7 cell line

The expression of a surface TCR/CD3 complex on the *Xenopus* B3B7 thymoma cell line implies rearrangement of both the α and β loci and the production of mature heterodimers. The gene of the TCR α chain has recently been cloned by Haire et al, (Haire,R.N et al, direct Genbank submission, 2001 acc# AF440803-AF440817). The α chain can be visualized in immunoprecipitation experiments as an acidic protein of 35kDa [1]. TCR β rearrangements in B3B7 were isolated by RT PCR. Two productive rearrangements (BV1s2/J2/D2/C and BV2/J3/D?/C) were isolated from this cell line as well as an out of frame BV12 rearrangement with a portion of BV4 fused to its 5' end. This aberrant event may be due to expression of RAG1 and 2 in this cell line (Louis Du Pasquier, personal communication, [21]. B3B7 does not therefore show allelic exclusion at the TCR β or Ig loci (Louis Du Pasquier, personal communication, [21]). The presence of three rearrangements may be due to the aneuploidity of the B3B7 line, which is not fully clonal and rearrangements continue at both Ig and TCR loci. Therefore, one cannot rule out the possibility of other rearranged TCR β chains in this cell line.

3.3.7 Tumor Immunity

Although T cells have been implied in *Xenopus* tumour immunity, tumour infiltrating lymphocytes have not been visualized. LG15 frogs are susceptible to ovarian tumours. Here, for the first time in *Xenopus*, we observe tumour infiltrating lymphocytes (TILs) in a naturally occurring tumour (figure 3.9). Some of these TILs stain very weakly for CD8 (data not shown).

3.4 Discussion

The exquisite conservation of this ITAM containing cytoplasmic domain provides us with an opportunity to study many new populations of T cells in *Xenopus* as well as delve into the functional makeup of the TCR complex in a poikilothermic vertebrate.

3.4.1 Is the conservation of the proline rich domain significant?

The ITAM in the cytoplasmic domain of CD3ε is not sufficient to account for the conservation of this region for ITAM containing regions of the other CD3 chains are not as well conserved [9]. This proline-rich region may play a role in interactions with proteins possessing SH3 domains [1, 22].

3.4.2 Advantages and problems of using this reagent

Ironically, it is the characteristic staining of SH3 domains which has limited the use of antibodies specific for this epitope. Many other proteins contain proline rich SH3 binding domains as a simple BLASTp search of the Genbank will show. By limiting the duration of the staining and the amount of antisera, a specific CD3ε staining can be obtained. However this reagent can recognize a multitude of intracellular cytoplasmic proteins containing proline rich regions. Therefore, this antiserum is not well suited to preparative immunoprecipitation from which TCRα or CD3ε chains could be sequenced. This is not an artifact of the polyclonal serum for the mAb also co-immunoprecipitates many unspecific (non-CD3ε) products (figure 3.3).

3.4.3 Which cell types will this reagent potentially stain?

CD3ε protein is present in the cytoplasm of fetal human NK cells and in chicken NK cells [23-26] therefore this reagent may help uncover previously unknown NK populations in *Xenopus* [1]. Populations of intraepithelial lymphocytes, which reside in the gut, are also generally CD3ε+ [1]. It is unclear what cell type the CD8^{hi} CD3εlo/- cells detected in the gut of adult *Xenopus* represent. However, in mice some populations of dendritic cells found in the thymus and spleen can be CD8 αα positive [27]. These authors did not look for any similar GALT associated dendritic cells.The brain sometimes showed small localized foci of staining (data not shown), but this did not seem to be as cellular as staining in other cell types. This reagent is known to recognize a population of Purkinje cells in the cerebellum which express CD3ε$^+$ therefore the same may be true in *Xenopus* [28].

3.4.4 Will this reagent prove useful for work on fish and elasmobranches?

This reagent recognizes CD3$^+$ homologues in birds (duck, chicken), an amphibian (*Xenopus*) and a chondrostean fish (sterlet) [1, 7, 10, 16]. However, similar experiments in trout, catfish (Gōbel and Hansen, unpublished as cited in [1]) have failed to detect a CD3ε chain in these species. This could be due to a fundamentally different TCR complex in these animals or to significant changes/differences in the cytoplasmic domain. The flounder sequence, published recently, contains a CD3ε chain lacking the conserved motif present in the intracellular domain [1,29]. However, a recent report suggests that some teleosts may possess this motif. A subset of peripheral blood lymphocytes in the snapper (*Pagrus auratus, Sparidae*) does stain with the rabbit polyclonal serum used in these *Xenopus* studies [30].

3.5 General Conclusions

With the mAbs available, three lymphocyte populations could be sorted (1) Ig$^+$ B cells which were all CD3$^-$, (2) CD8$^+$ Ig$^-$ T cells which all reacted with the CD3ε antiserum, the CD8$^-$Ig$^-$ population included some CD3$^+$ cells which most likely represent the CD4$^+$ cells, and (3) CD3$^-$ cells which probably represent other leukocytes present in the lymphoid gate.

An anti-*Xenopus* CD4 mAb would help to better characterize these subsets. The CD3ε antiserum also stained discrete lymphoid cells, some of which co-expressed the CD8 molecule. Although it is not known for *Xenopus laevis*, the CD3ε antiserum may also react with NK cells as is the case in mammals and chickens where NK cells express cytoplasmic CD3ε [23-26].

The *Xenopus laevis* derived thymoma cell line B3B7 also stained with the CD3ε antiserum which led to the first formal proof of cell surface expression of the TCR on the B3B7 cell line. Previously, B3B7 has been shown to express the CD8αβ heterodimer [11, 20]. The use of these antibodies to CD3ε for preparative immunoprecipitation of proteins of the TCR complex does not seem viable, however, the B3B7 cell line may still play an important role in the large scale isolation of T cell proteins as more immunoreagents become available. The gene of the TCR α chain has recently been cloned [31], therefore the only chain in the TCR left to be cloned is CD3ε. This polyclonal serum may be able to purify CD3ε efficiently under harsher lysis buffer conditions.

No Text

```
peptide                           ERPPPVPNPDYEP
human    KNRKAKAKPVTRGAGAGGRQRGQNKERPPPVPNPDYEPIRKGQRDLYSGLNQRRI NP_000724
mouse    KNRKAKAKPVTRGTGAGSRPRGQNKERPPPVPNPDYEPIRKGQRDLYSGLNQRAV A31348
cow      KSRKAKASPMTRGAGAGGRPRGQNKGRPPPVPNPDYEPIRKGQRDLYAGLNQRGV JC4663
pig      KSRKAKAMPVTRGAGAGGRPRGQNRERPPPVPNPDYEPIRKGQRDLYSGLNQR   AAB46744
sheep    KSRKAKATPMTRGAGAGGRPRGQNRERPPPVPNPDYEPIRKGQRDLYSGLNQRGV P29328
rabbit   KNRKAKCKPVTRGAGAGGRPRGQNKERPPPVPNPDYEPIRKGQRDLYSGLNQRGI BAA86993
dog      KTRKANAKPVMRGTGAGSRPRGQNKEKPPPVPNPDYEPIRKGQQDLYSGLNQRGI P27597
wallaby  KARKAKAKPVGRGGGGGGRTRGANKERPPPVPNPDYEPIRKGQRDLYAGLNQRAI AY028923
chicken  KNKKGQSR-----AAAGSRPRAQKMQRPPPVPNPDYEPIRKGQRDVYAGLEHRGF CAA70120
duck     KDKKGRPS-----AGAGSRPRGQKTQRPPPVPNPDYEPIRKGQREVYAGLESRGY AF378704
sterlet  QNRKG-------ASAMAPAARPGRQNRAPPVPNPDYEPIRTGNREVYSGLNKRT  AJ242944
flounder HKKIRL------SSGSERPLPNEMRNRASNDPYQRLRFNSGARKDTYDVINHNR  AB054068
```

Figure 3.1 Conservation of a cytoplasmic CD3ε epitope

An alignment of the cytoplasmic domains of known CD3ε homologues with a synthetic peptide used in the generation of a rabbit anti-human CD3ε serum reveals a highly conserved proline rich region. The alignment was generated by Clustal software using a PAM 250 residue weight matrix. The shaded conserved region contains several motifs important for TCR/CD3 assembly and signal transduction, including a potential adapter site for SH3 domains and the ITAM motif [4]. This diagram is modified from [1].

NO Text

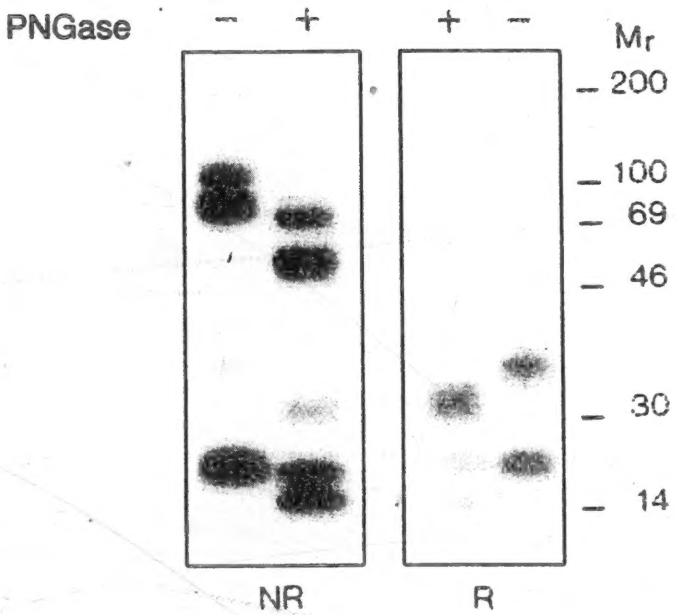
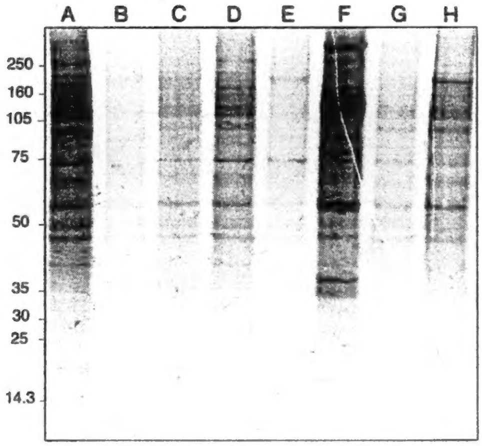


Figure 3.2 The *Xenopus laevis* αβ-TCR is associated with at least two CD3 proteins

Iodinated *Xenopus* B3B7 thymoma cells were lysed with digitonin and immunoprecipitation was carried out with the polyclonal CD3ε antiserum. PNGase F digestion to remove N-linked carbohydrates was performed as indicated. The lysates were analyzed under nonreducing (NR) and reducing (R) conditions on a 4-20% PAGE gel. Identical results were obtained using freshly isolated *Xenopus* splenocytes (data not shown). The CD3ε antiserum specifically immunoprecipitates proteins species of 110 kDa, 75 kDa, and 18 to 20 kDa under non-reducing conditions and 40 kDa, 35kDa and 18 to 20 kDa proteins under reducing conditions. Further analysis suggests TCR heterodimers (75 kDa) are associated with CD3ε (18 kDa) and CD3γ/δ (20 kDa) chains [1]. Two dimensional electrophoresis showed TCR heterodimers are composed of acidic and basic components which represent the α and β chains respectively [1]. An additional 110 kDa band may represent a covalently linked multimer of an αβ-TCR dimer linked to a CD3 dimer [1]. This experiment and experiments described here were performed by Thomas Göbel.

Figure 3.3 The monoclonal antibody and polyclonal antiserum are equivalent

^{35}S labeled B3B7 cells were lysed and immunoprecipitation carried out and lysates seperated on a 4-20% SDS-PAGE gel under reducing conditions. The lanes are as follows: (A) anti-rat Ig, (B) rat anti-CD3ε monoclonal first immunoprecipitation, (C) rat anti-CD3ε monoclonal second immunoprecipitation from same lysate as for (B), (D) anti-rabbit Ig, (E) rabbit anti-CD3ε polyclonal, (F) unspecific antibody (chicken 3-6-3) on Sepharose beads, (G) anti-mouse Ig, (H) mouse anti-XT-1 monoclonal antibody. After coimmunoprecipitation with anti-CD3ε we expect bands of 40 kDa, 35kDa and 18 to 20 kDa under reducing conditions. Lysis conditions needed to maintain the integrity of the TCR complex for coimmunoprecipitation allow many unspecific protein-protein interactions as well.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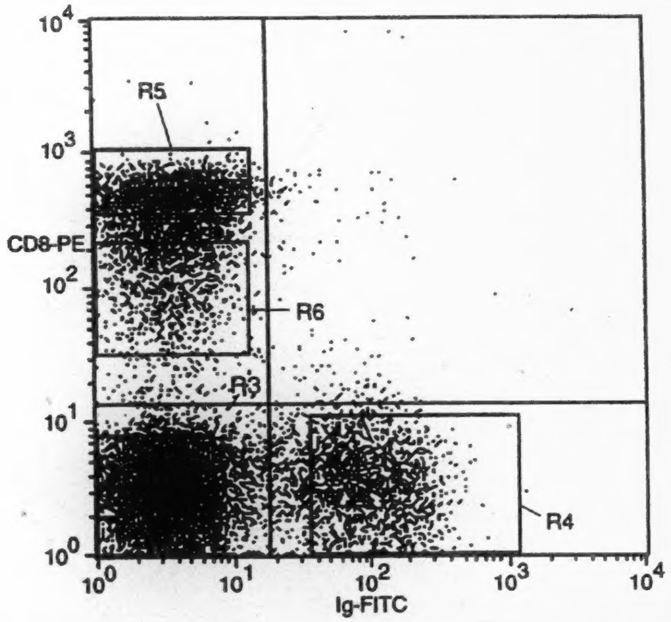


Figure 3.4 Fluorescence activated cell sorting of *Xenopus* splenocytes Single cell suspensions of *Xenopus laevis* splenocytes were double stained with AM22 (anti-CD8) and 10A9 (anti-IgM) monoclonal antibodies, followed by *Xenopus laevis* red blood cell (xRBC) adsorbed PE conjugated anti-mouse IgM and FITC conjugated anti-mouse IgG1. Cells were sorted on a FACs Vantage cell sorter according to the intensity of their staining. Each dot on the graph depicts a single cell. On the x-axis is the staining for IgM (specific for B cells) and on the y-axis the staining for CD8 (specific for T cells). Cells falling into the four boxed areas (gates) were sorted into separate vials for further analysis (see figure 3.5 below). Gates were determined by measuring control preparations of unstained cells and cells stained with only the secondary labeled antibodies. Gate R3 contains CD8$^-$ IgM$^-$ cells which include monocytes and CD4 T cells and $\gamma\delta$ T cells as well as other cell types from the thymus that do not stain with these antibodies. Gate R4 contains B cells which are CD8$^-$ and IgM^{hi}. The remaining cells fall into the IgM$^-$ gate but were sorted into a CD8^{hi} (R5) and CD8^{lo} (R6) populations. Note that there are very few cells that fall into the IgM$^+$ CD8$^+$ gate and these constitute background.

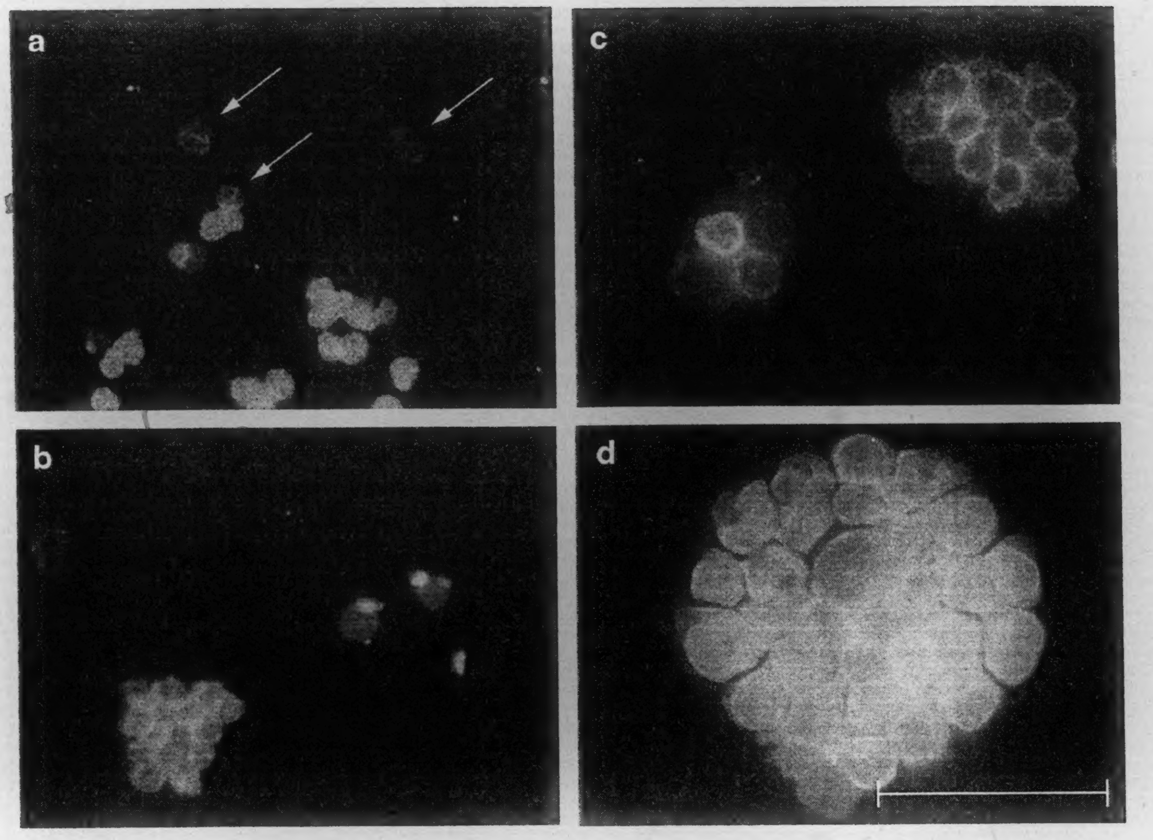


Figure 3.5 CD3ε staining of *Xenopus laevis* splenocytes and the B3B7 thymoma

Cytospin preparations of (a) Ig$^-$ CD8$^-$, (b) Ig$^+$ CD8$^-$ and (c) unsorted splenocytes were stained with the CD3ε antiserum. Arrows indicate examples of negative cells. (d) The *Xenopus* derived B3B7 thymoma cell line was labeled using the CD3ε antiserum (green) and propidium iodide (red). Scale bar represents 100 µm. (Taken from work I contributed to [1]).

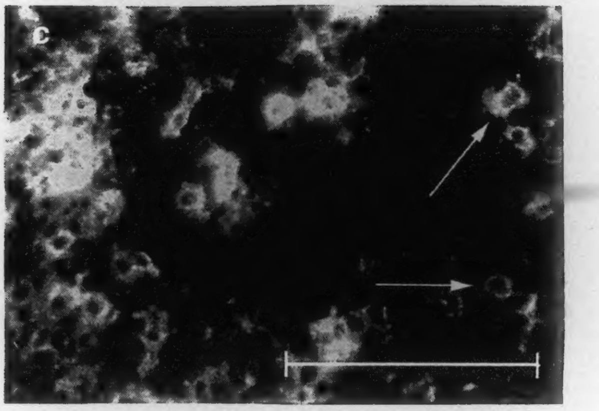
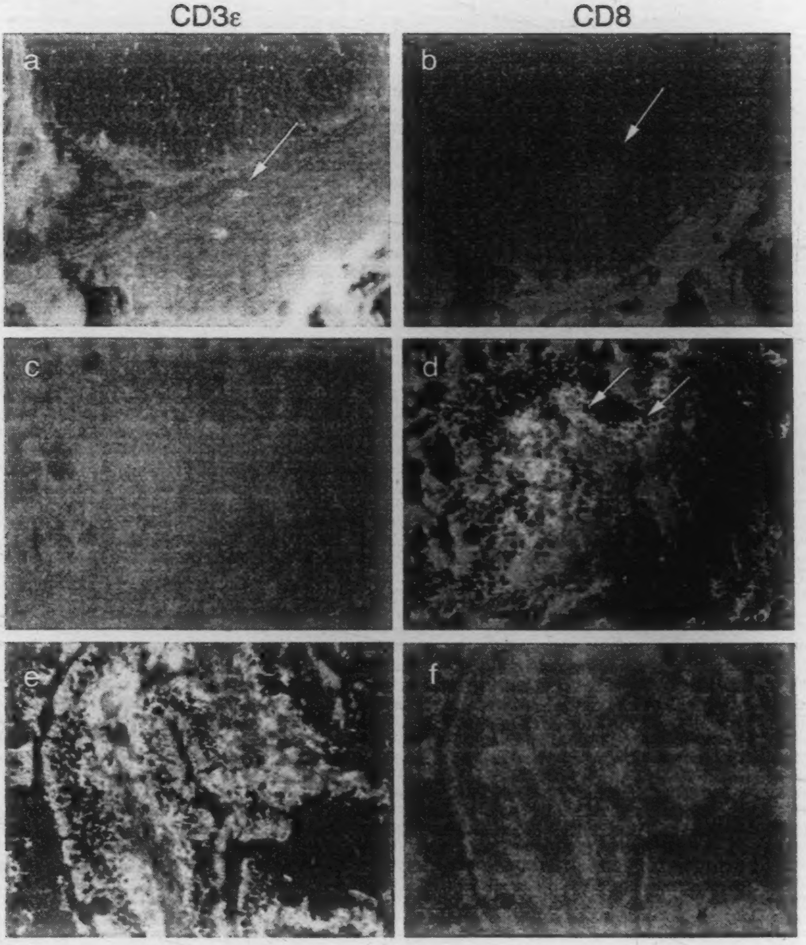


Figure 3.6 T-lymphocytes are specifically stained with CD3ε antiserum the *Xenopus laevis* spleen

Tissue sections of spleen from adult outbred frogs was stained with the CD3ε antiserum (green) and an anti-CD8 mAb (red). Double labeled CD8$^+$ CD3ε$^+$ cells appear as yellow cells. A control section was stained with the secondary antibody only and found to be negative. Examples of CD3ε single positive and CD3ε$^+$CD8$^+$ double cells are indicated with arrows. (Taken from work I contributed to [1]).

Figure 3.7 T-lymphocytes are specifically stained with CD3ε antiserum in *Xenopus laevis* tissues

Tissue sections of intestine from adult outbred frogs were stained with the CD3ε antiserum and an anti-CD8 mAb. For each experiment a control section was stained with the secondary antibody only and found to be negative. (a, b) the arrow indicates a CD8^{+} CD3ε^{+} cell in smooth muscle. Several other cells are CD3ε^{+} only. (c, d) GALT region rich in T cells. (e, f), (g,h), (i,j) and (k,l) show other representative stainings of different regions of the adult intestine. Arrows in (d), (j) and (l) indicate CD8^{hi} CD3$\varepsilon^{lo/-}$ cells. These cells are rare and it is not clear what cell type they represent.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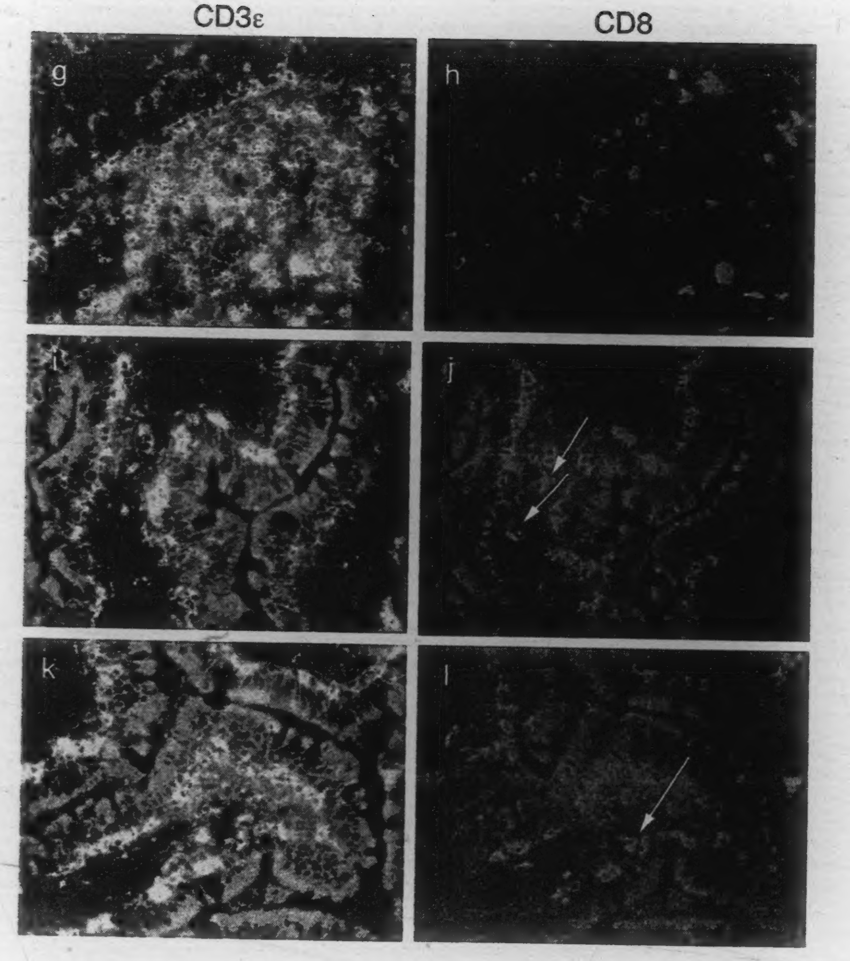
CD3ε
CD8
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j
k
l

Figure 3.8 Anti-CD3ε staining of tadpole tissue sections

The CD3ε expression was analyzed using (a) thymus, (b) liver, (c) stomach, (d) spleen, (e) intestine, and (f) skin. All tissues were obtained from 7 day old tadpoles except spleen which was obtained from a 9.5 day old animal. Arrows indicate CD3ε$^+$ cells. Propidium iodide (red) was used as a counterstain in sections b, and f. Control sections (g, h, I) incubated with only secondary antibody were negative. In (g), L represents the tadpole liver and s the skin. Sections (h) and (I) show the intestine and thymus, respectively. Scale bar represents 100 μm. (Taken from work I contributed to [1]).

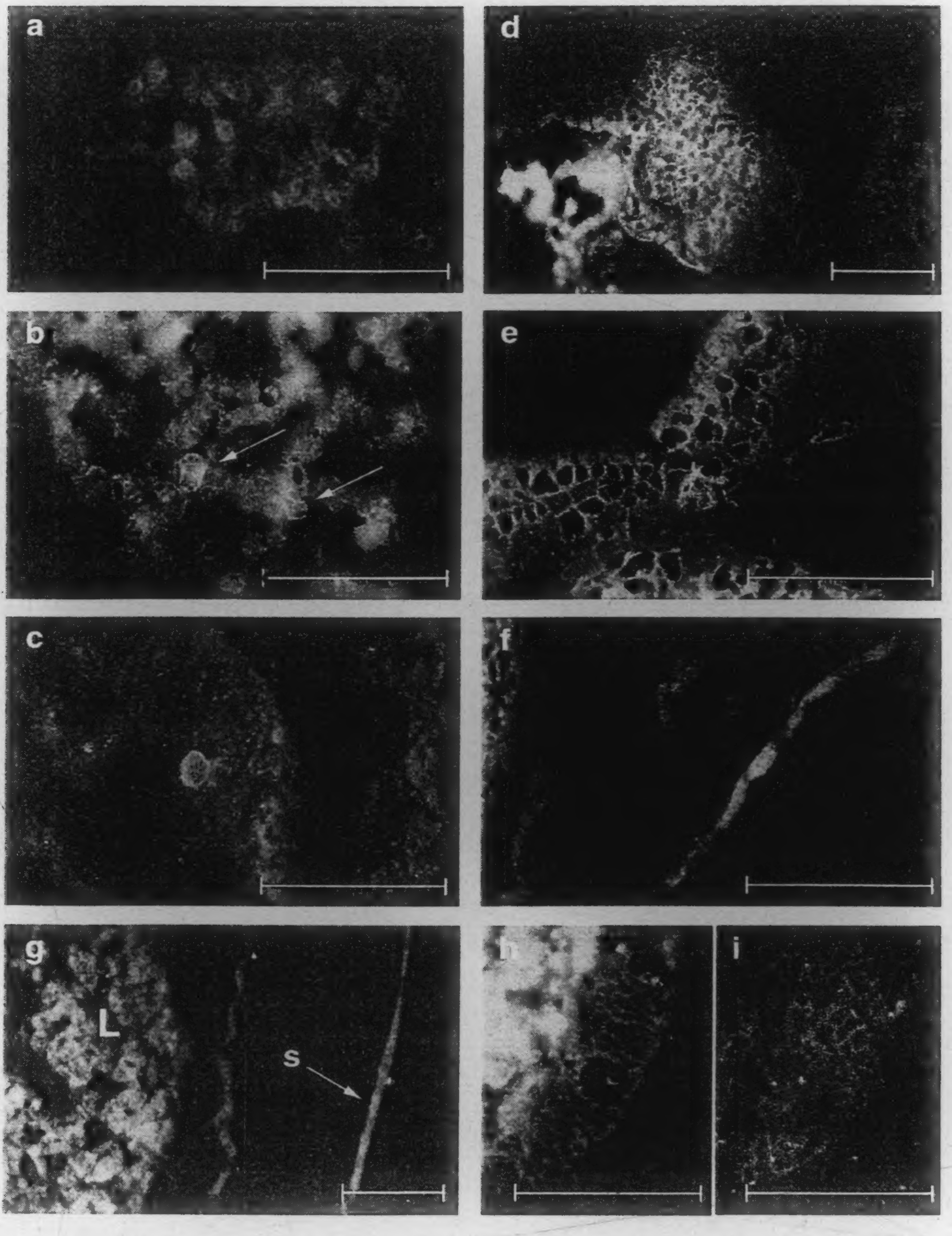
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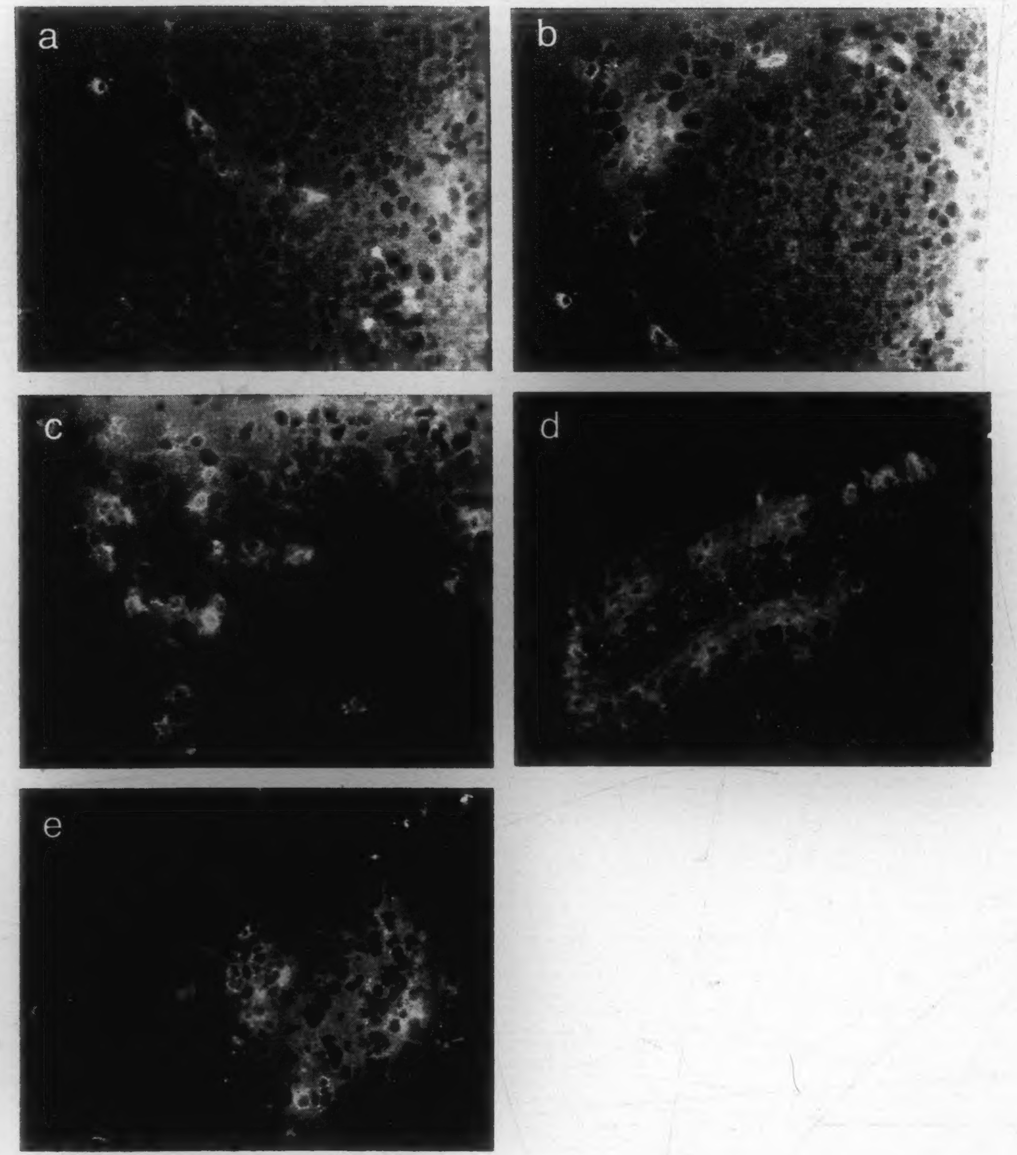
Figure 3.9 Some tumour infiltrating lymphocytes (TILs) are CD3ε$^+$

LG15 frogs are prone to ovarian tumours. Sections taken from these tumours show CD3ε$^+$ cells around the margins of the tumour capsule (a, b, d) or infiltrating the masses (c, e). Occasionally, these TILs stained very weakly with AM22 (putative CD8α) (data not shown) but never CD8 high or intermediate. In several cases aggregations of CD3ε$^+$ cells were found (b, c, d, e). It is unclear if this represents an interaction of distinct cells or an expansion of a single T cell clone.


a
b
c
d
e

L

No Text

References

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Chapter 4

T cell Ontogeny in *Xenopus*

Notext

4.0 T cell ontogeny in *Xenopus*

4.1 Introduction

The time point of first exposure to the outside world and foreign antigens differs widely among jawed vertebrates. Whereas mammals and birds are physically well protected within the womb or the egg during early development, many amphibians and fish hatch from their egg casings relatively soon after fertilization. Marsupials, which are born at a less developed stage than placental mammals, also differ from the mouse and human examples.

Exposure to the outside world at a relatively early point in development would provide a considerable evolutionary pressure to develop the capacity to make immune responses early in development. If the adaptive immune system plays a significant role in early immune responses, we would expect to find B and T cell production at time points well before that of birds and placental mammals. Furthermore, in animals that are quite small at the time of hatching, the limited number of T and B cells may affect the ability to mount adaptive immune responses more than genetic or recombinational constraints. One would predict that in order to maximize the usefulness of a small number of cells the population should have many cells, all with different receptors (polycolonal) rather than a few T cells which have proliferated, producing many cells with only a few receptor specificities (oligoclonal).

Xenopus tadpoles, which hatch from their egg casings two days after fertilization, provide a good model in which to test these assumptions. Previous studies have defined *Xenopus* T cell ontogeny in terms of immune responses and histological or immunohistological observations. Thymectomy (Tx) has been used extensively to probe the need for the thymic environment during development as well as the role of T cells in *Xenopus* immune responses. The work presented in this chapter extends our understanding of the ontogeny of *Xenopus* T cells by allowing us to better define early T cell populations and determine the onset of transcription and somatic DNA recombination at the TCR β locus in tadpole life.

In order to put these findings into a proper context we must review the ontogeny of T cells in other animals.

4.2 The ontogeny of $\alpha\beta$ T cells in other vertebrates

4.2.1 Skate

The TCR and TdT genes are upregulated in the thymus at week 8 of embryogenesis. TCR expression in the body periphery is found in hatchlings and adults [1].

4.2.2 Rainbow trout

RAG expression is first detected in the trout at 10 dpf, before the thymus and pronephros are apparent (as reviewed in [2]). TdT, as detected by RT-PCR, was present before hatching, and this differs from other studied species where the first TdT expression coincides with either birth, hatching, or in the case of *Xenopus*, the completion of metamorphosis (as reviewed in [2]). Complete VDJ rearrangements are first detected at 15 dpf, and do not appear to contain N insertions, whereas VDJ junctions at 4 days post hatching did contain putative N insertions [2].

4.2.3 Reptiles

No information is currently available for TCR in reptiles.

4.2.4 Axolotl

The adaptive immune response in urodele amphibians (salamanders and newts) occurs much more slowly than in anuran amphibians (as reviewed in [3]). Axolotls (*Ambystoma mexicanum*) hatch from their egg casings 3 weeks postfertilization. The thymus and the spleen form around 4 weeks postfertilization (as reviewed in [4]). The first RAG mRNA is detected at 6 weeks postfertilization in the head (thymus) and body (liver and spleen) [5], but it is not until 10 weeks that one can detect full Ig or TCR rearrangements [6-8]. TdT is not expressed at the time of the earliest rearrangements, only being detected at 13 weeks (as reviewed in [2]).

As we have seen in chapter 2, the axolotl possesses a substantial number of genomically encoded TCR β segments and these are as variable as those of mice or humans [9-11]. However, this available diversity does not translate into robust T cell responses.

The axolotl adaptive immune response is notoriously slow when compared to *Xenopus* and other anuran amphibians (as reviewed in [4]). Graft rejection takes much longer in urodeles, and *in vitro* responses to foreign cells are less robust than in anurans or mice [12-14].

4.2.5 Chickens

T cell precursors enter the thymus on day 7 postfertilization embryonic day 7 (E7). The first αβ T cells (TCR2) emerge on day 15 [15, 16]. TCR2 cells are first seen in the thymus by embryonic day 19 (E19) just two days before hatching on day 21 post-fertilization [17-19]. TCR 2 cells can be divided into those which use family Vβ1 and those which use Vβ2. During embryonic development Vβ1 is rearranged before Vβ2. This sort of developmentally programmed TCR β rearrangement has not been observed in other vertebrates studied to date [20].

4.2.6 Mice

In mice, T cell precursors enter the thymus between days 10 and 11 of gestation [21]. D-J rearrangement at the β locus commences on day 14, although D-J rearrangements are sometimes observed in T cell precursors in the murine fetal blood before thymus colonization. Full rearrangements are first detected on fetal day 14 [22-25]. TCR β expressing cells are first detected on gestational day 17 when the rearranged TCRα loci begins to be expressed, but only on day 19 do substantial numbers of thymocytes start expressing TCR β [26-28]. Mature αβ T cells, as defined by full TCRβ rearrangements, and surface expression of CD3ε, and either CD4 or CD8 are only present after birth [27, 29, 30]. By fetal day 18, functional allospecific CTLs and the first helper T cells are present in mice but these do not reach functionally relevant numbers until after birth on day 19 [31].

4.2.7 Humans

The human thymic epithelium forms by 6 weeks from the third and fourth pharyngeal pouches and the first precursor lymphocytes enter the thymus by 8 to 9 weeks. The thymus is divided into a cortex and medulla by 10 weeks [32] suggesting that the organ is mature enough to support T cell development. Humans possess thymocytes with a mature phenotype during fetal life. $\alpha\beta$ T cells are present in the human thymus [33] and CD4, CD8 and CD3 markers are present on the surface of thymocytes by 12 weeks [34, 35]. By the end of the second trimester, human fetal thymocytes employ a full Vβ repertoire [29], using all 20 Vβ families. T cells are present in the periphery before 15 weeks in the spleen and before 20 weeks in the lymph nodes [36]. Allogeneic antigen specific cell mediated cytotoxicity is present by 18 weeks [37] and thymocytes from 24 week fetuses can proliferate when PHA stimulated [32]. Plasma cells (activated B cells which secrete antibodies) have been documented in 20 week fetuses with congenital syphilis [36]. These results show that T and B cell responses can occur before birth in some mammals.

4.2.8 Marsupials

Marsupials are born in a less developed state than their eutherian counterparts and are therefore less protected from potential pathogens during early development. From this perspective, they are more like frogs and fish which are exposed to antigens early in development. Then length of gestation varies from species to species, ranging from 11 to 32 days post-fertilization. Marsupials are, at birth, immunoincompetent and possess no mature lymphoid tissues. Like other mammals, the thymus is the first lymphopoietic organ and in some species CD3ε^+ cells can be detected on day 2 postpartum. Despite this, a cortex and medulla do not form until several weeks later. There are conflicting experimental results concerning the onset of immunocompetence in many marsupial species. These conflicts may stem from some peculiarities of the marsupial adaptive immune response. Although T cells proliferate when in the presence of classical T cell mitogens (PHA, ConA), there is a little, if any, *in vitro* alloresponsiveness measured in all marsupial species studied to date [38].

4.3 T cell ontogeny in the *Xenopus*

Functional and histological studies have been used to define T cell ontogeny in *Xenopus*. A summary of the ontogeny of the adaptive immune response in *Xenopus* is given in figure 4.1.

4.3.1 Are tadpoles the fetal or the neonatal equivalent?

Before discussing T cell ontogeny in *Xenopus*, it is necessary to discuss what constitutes a fetal animal, a neonate or an adult. The situation is straightforward in mammals, where the fetus is protected in the uterus. In birds, the developing chick is referred to as an embryo [39]. After hatching or birth, the animal enters a short neonatal period that gradually progresses into an adult situation. It is unclear however whether one can directly compare tadpoles to developing birds and mammals. Both frogs and fish spend varying amounts of time in an egg casing. Upon hatching, they continue to grow. In fish, this progression leads to an adult form, therefore fish development may be described as a very short embryonic stage followed by a neonatal and adult form much like that of birds and mammals.

Xenopus tadpoles could also be considered, by some, as a form of neonate when it hatches after two days of embryonic life in the egg casing. However, unlike other vertebrates, amphibians undergo metamorphosis to pass into adulthood. In terms of the adaptive immune system, there are many differences between tadpole and adult immune responses. Tadpole cells are qualitatively different from adult cells in their reactivity. Thus, it is difficult to determine what stage of mammalian life cycle the frog tadpole is most similar to. In keeping with convention and because, like embryonic mammals and birds, tadpoles lack TdT expression, in this work, tadpoles will be interchangeably referred to as larval and animals still within their egg casings will be referred to as embryos.

4.3.2 When do mature T cells first leave the thymus?

Studies using thymectomized *Xenopus* suggested that mature T cells begin to leave the thymus on approximately 7-8 dpf. The removal of the thymus (thymectomy) on days 4-8 post-fertilization severely limits allograft (graft from an animal of the same species expressing at least one different MHC antigen) rejection; responses to T cell dependent antigens and interferes with Ig Y (analogue of mammalian Ig G) antibody responses [40, 41] as reviewed in [42]. Such humoral responses require help from (interaction with) class II restricted T cells (as reviewed in [40]). Thymectomy (Tx) on day 7 or 8 post-fertilization does not always lead to a complete loss of T cell dependent responses suggesting that T cells have left the thymus and entered the periphery of the body at this time point [41, 43, 44]. This is supported by the observation of a rapid expansion in the number of B cells between 9 and 14 dpf which might be attributed to the presence of active, functional peripheral T cells producing cytokines [45, 46]. Functional and cellular studies suggested that the first functional *Xenopus* T cells must develop and enter the periphery between 4 and 12 dpf.

4.3.3 Expression of T cell markers during ontogeny

The study of *Xenopus* T cell subsets has been difficult due to the small number of T cell specific monoclonal antibodies and antisera available. However, some reagents are available and T cell ontogeny has been further defined by immunohistological studies of the expression of several putative T cell markers. Cells positive for XT-1 (present on a subset of *Xenopus* T cells), CD8 (AM22) and CD5 (T cells and activated B cells in *Xenopus*) are not detected before day 7 (~ stage 48) [44]. Unlike late tadpole thymocytes, only 20% of 7 dpf thymocytes are XT-1 suggesting that the thymus is still in a relatively immature state. AM22$^+$ (putative CD8α chain) cells are first detected in the thymus on day 8 and are occasionally found in day 12 spleen. CTX, a marker expressed on cortical thymocytes, is first detected by immunofluorescence on day 8. These cells are thought to represent the *Xenopus* equivalent of the CD4CD8 double positive (DP) thymocytes [47, 48]. In mammals the DP subset of thymocytes is actively undergoing T cell selection [49]. A paucity of CD8$^+$ peripheral T cells is expected in tadpoles, because, unlike adult *Xenopus* and other studied vertebrates, tadpoles lack MHC class Ia and Ib expression [50] (see chapter 1 section 1.3.3.1.2). Indeed, only 5% of splenocytes in day 7 (~stage 48) tadpoles were CD8$^+$ as compared to the adult spleen where 30% of the cells were CD8$^+$. The lower number of peripheral CD8$^+$ T cells in tadpoles seems due to thymic selection, as 70-80% of thymocytes in 12 dpf (stage 49) animals are CD8$^+$ and CTX$^+$ (putative unselected thymocytes [47, 48]) whereas CD8$^+$ cells are only rarely detected at this stage in the spleen [44].

Taken together, these results showed that the first T cells left the thymus on day 7, 2 to 3 days after the thymus is first colonized by T cell precursors.

4.4 A new approach to *Xenopus* T cell ontogeny

Many previous studies have helped outline T cell ontogeny in early *Xenopus* life. However, immunohistological reagents available may be insufficient to detect all T cell subsets such as γδ T cells or intraepithelial lymphocytes. Furthermore, it is not clear when T cells begin to rearrange their TCR loci or if this process is strictly thymus dependent.

Using a newly available antiserum to a conserved intracellular CD3ε epitope (chapter 3) it has become possible to observe the ontogeny of T cell subsets in tadpoles which have not been characterized in *Xenopus* before. In addition, the recent cloning and characterization of *Xenopus* TCR β segments (chapter 2) makes it possible to monitor the onset of transcription and rearrangements at the TCR β locus in tadpole T cells. In this chapter, the onset of rearrangement at the TCR β locus is defined and these early rearrangements are studied especially with regard to their usage of Vβ segments. Finally the length of the CDR3 junctions in adult and tadpole TCR β chains is compared.

4.5 Materials and methods

4.5.1 Animals and strains

Hybrid LG15 animals were produced by gynogenesis as previously described [52]. Animals were staged according to the Nieuwkoop and Faber table

4.5.2 cDNA preparations

Thymus and spleen were dissected using separate sets of forceps to minimize cross-contamination risks. RNA was extracted using Trizol (GibcoBRL) according to the manufacturers protocol except 15μl of water per 1ml Trizol was added to the initial homogenization step and glycogen was used as a carrier in all precipitations. Because of the paucity of T cells in early tadpole development, organs from 8-10 genetically identical tadpoles were pooled. 500 to 1000 ng of total RNA was reverse transcribed as previously described [54] (see appendix A).

4.5.3 RACE PCR

RNA was isolated from anterior (head) and the posterior (body) of 60, 72 and 84 hour post-fertilization tadpoles using Trizol as per above protocol. The anterior/posterior cut was made with a new sterile scalpel blade and the two sections manipulated by separate forceps. 1μg of total RNA was used to generate 5′ RACE libraries using the Smart RACE kit (CLONTECH) according to the manufacturers instructions except that random hexamer primers were added to prime the first strand synthesis in addition to the given anchored poly-T primer and that the adaptor oligonucleotide was added after 2 minutes of cDNA synthesis (see appendix A). 1μl undiluted template RACE cDNA was amplified with a sense adaptor specific primer (CTAATACGACTCACTATAGGGC or AAGCAGTGGTAACAACGCAGAGT) (CLONTECH) and an antisense Cβ primer (ATTCTTGGTGAGGCTGAGGCGGCTGC) using Li^+ based dNTPs, Taq DNA polymerase and Perkin-Elmer 10X PCR buffer in a volume of 50μl. PCR was carried out on a Techne thermocycler as follows: 5 cycles of 94°C 10sec, 68°C 10 sec, 72°C 2 min, 5 cycles of 94°C 10sec, 65°C 10 sec, 72°C 2 min, and finally 25 cycles of 94°C 5sec, 63°C 10 sec, 72°C 2 min followed by 10 minutes at 70°C. 1 μl of a 1:75 or 1:45 dilution of this initial reaction was reamplified in a 30μl volume using Vβ specific primers and a (C_G/C_U) consensus Cβ antisense primer as follows: 35 cycles of 94°C 15sec, 57.5°C 45 sec, 72°C 1 min followed by 10 minutes at 70°C followed. PCR products were then separated on a 1% agarose gel and bands larger than 450 bp were purified using Geneclean (BIO 101). This population of products was ligated into the PCR II dual promoter plasmid using the TA cloning kit (Invitrogen).

4.5.4 Multiplex PCR

Because of the paucity of *Xenopus* T cells at the stages when rearrangement of the TCR β chain starts in the thymus (roughly 100 lymphocytes on day 6), multiplex RT-PCR was employed (see figure 4.7). Thymi from 8-10 tadpoles were pooled for each cDNA preparation. A first round of PCR employs multiple Vβ primers and a single constant region primer. After 20-25 rounds of amplification the reaction is split into separate reaction tubes, each containing a nested primer for a single V and a constant region primer. The second round of PCR is carried out and Vβ presence or absence assayed on an agarose gel or by sequencing. Great care was taken to keep PCR conditions uniform and to prevent contamination. Master mixes for each of the Vβs were prepared and split into positive (internal Cβ and adult thymus) and negative controls (water controls for both the first and second rounds of PCR and egg or cDNA from day 2 whole embryos) as well as for the conditions tested. A subset of the products were transferred to filters and filters were hybridized by Cβ probe or Vβ specific probes to ensure the fidelity of the primers under these amplification conditions.

Where positive controls from older animals were performed they were pipetted from the same master mix after the other tubes were closed.

5.4.5 Subcloning

PCR products were purified on spin columns (QIAGEN), ligated into PCR II dual promoter or PCR 2.1 vector (Invitrogen), and transfected into chemically competent TOP 10 F' bacteria (Invitrogen). Ligated PCR product was then transfected into DH10 *E. coli* bacteria by electroporation as previously described [54]. Bacteria were plated out onto complete medium agarose plates with Ampicillin, X-Gal and IPTG and grown at 37°C overnight, and stored at 4°C.

4.5.6 Sequencing reactions

Sequencing was carried out on both ABI and LI-COR systems. Sequences were then compared to a local sequence database using software developed by Stefan Meyer based upon the BLAST algorithm. Sequence alignments were generated using either the Lasergene multi-sequence alignment tool or CLUSTAL using a PAM 250 matrix. Genbank accession numbers for previously submitted sequences can be found in the series U60424 to U60436 and U75994.

4.5.7 Hybridization of Genomic DNA

PCR products were separated on 1.0% agarose gels, and transferred to Zeta-Probe membranes (BIO-RAD) via alkaline transfer using standard protocols [55]. High stringency hybridizations were carried out with a Cβ domain probe at 65°C for 16-24 hours and then washed at 65°C in 0.1x SSC and 0.1% SDS for 30 min and auto-radiographed using BIOMAX MR films (KODAK).

4.5.8 Histology

Tadpoles were fixed in 4% paraformaldehyde, embedded in plastic resin and cut into 6μ sections. Sections were stained with Modified Nocht's Azure-eosin stain (0.1% azure A 8.0 mL, 0.1% eosin B 8.0 mL, 0.1M citric acid 1.2 mL plus 25mL water. pH to 5.2 with disodium phosphate and add 5.0 mL of acetone).

4.5.9 Immunohistology

Whole tadpoles, adult tissues and tumors were frozen in O.C.T. embedding medium (Tissue Tek), cryosectioned (6μ), fixed 1 minute in acetone, and blocked with 1.5% BSA in PBS. A 1:25 dilution of the rabbit anti-human CD3ε antiserum (DAKO A0452) was adsorbed twice volume to volume on *Xenopus* red blood cells. The adsorbed serum was further diluted 1 to 100 and incubated with the sections for 2 hours at room temperature. Sections were washed 3 times in PBS and again blocked followed by a 30-60 minute incubation with FITC or TR labeled goat-anti-rabbit Ig (SBA, Birmingham, AL) antibody (1:200 dilution) (Southern Biotech). Anti-CD8 staining was performed with the AM-22 mAb [56] (1:75 dilution) followed by a fluorochrome conjugate of goat-anti-mouse-IgM (SBA).

4.6 Results

4.6.1 The early ontogeny of the thymus

Although the entry of T cell precursors into the thymus has been well documented, it was necessary to determine if a dissection of the early thymus and of the early liver could be cleanly carried out. The sensitivity of PCR techniques requires an absolute separation. An estimation of the absolute numbers of cells present in the thymus also serves as a control of cell number for the interpretation of PCR results. Plastic embedded tadpoles were sectioned and stained with a dye which stains lymphocytes and other very metabolically active cells a deep purple (figure 4.2). On day 5, the thymus is, at most, 5 or 6 cells across and very few of these appear to be lymphocytes. In the following days the thymus grows rapidly and fills with lymphocytes. By day 7, the cortex and medulla structure of the thymus is apparent.

4.6.2 CD3ε staining in the early tadpole

To count T cells in a young tadpole and to see where they are localized we used the CD3ε antiserum characterized in chapter 3. Tadpoles were cryosectioned tangentially from the eyes to the base of the tail, and serial sections stained with the polyclonal CD3ε antiserum.

In mammals and chickens, CD3ε is expressed on the surface of αβ and γδ T cells as well as intracellularly in fetal human NK cells and chicken NK cells [57-59]. Therefore, in *Xenopus*, CD3ε^+ cells could represent both T and NK cells.

No CD3ε^+ cells were detected in two day 4 LG15 tadpoles. In one d5 tadpole, a few (6 or 7) lightly positive cells were detected in the lining of the stomach and the portion of the esophagus proximal to the stomach (figure 4.3). This staining was not detected in other day 5 tadpoles. By day 6, there were more cells in the esophagus (20), a similar number in the stomach (27) and several positive cells in the liver. By day 7, 825 CD3ε^+ cells are found in the skin, gut, liver and stomach as well as the thymus. Approximately 2000 CD3ε^+ cells were found in a day 9 tadpole. The number of gut-associated CD3ε^+ cells remained constant from day 7 to 9, whereas, in skin, thymus, and liver the number of positive cells in the day 9 tadpole was increased. Different staining intensities and cell morphologies were observed in all animals suggesting that several different cell types are CD3ε^+ in *Xenopus* tadpoles. There was considerable variation in lymphocyte T cell numbers in day 6, 7 and 9 tadpoles, even in animals at the same morphological stage.

By day 15.5, the thymus is well developed and has a distinct cortex and medulla.

Figure 4.4 shows CD3ε staining as compared to that of a cortex specific marker, CTX and a B cell specific antibody (10A9). The CD3ε staining is quite bright and uniform in both the cortex and the medulla. Note however that the outermost layers of the cortex are not well stained. As is the case in mammals, this area contains cells, which have not started to express RAG1 at a high level [49] or to undergo V-D-J rearrangement (figure 4.4, (d) and (e)). Therefore, CD3ε staining seems brightest on those cells that are undergoing or have undergone VDJ recombination. The thymus of younger animals does not stain as brightly with CD3ε , perhaps due to the immature state of the thymus or due to an extremely rapid passage of mature CD3ε^+ cells into the periphery. Further studies are needed to differentiate between these possibilities.

4.6.3 The onset of RAG1 transcription

Our next indication that TCRβ rearrangements might start very early in tadpole development came from the onset of RAG1 transcription. Although RAG1 transcripts were never found in 6h embryos, the majority of cDNA preparations from 48h embryos did contain some RAG1 message (figure 4.5). This is in agreement with the findings of Mussmann et al [45] who found RAG1 expression from day 3 onward. RAG2 expression was not measured for it lacks an intron to distinguish mRNA from contaminating genomic DNA during PCR studies, and genomic DNA contamination was found to be a problem with RAG2 primers (data not shown). The presence of RAG1 message was used routinely to gauge the quality of cDNA samples.

4.6.4 Transcription from the *Xenopus* TCRβ locus

TCRβ rearrangements were assayed for in RACE libraries prepared from the anterior (head) or posterior (body without tail) of 60, 72, and 80h pf tadpoles. RACE libraries, where an adaptor is added to the 5' end of cDNAs, allow the amplification of all Cβ containing cDNAs in a sample. For a detailed description of the RACE technique please refer to figure 5.1 and appendix A. A faint band amplified from the posterior of pooled 84h pf tadpoles contained two main products (data not shown). One was a sterile (untranslated) transcript of the *X. laevis* constant region (C_L) with 5' UTR sequence. The others were sterile transcripts consisting of the J13 segment spliced to C_L (see figure 4.6). J-C and D-J-C transcripts have also been found in older tadpoles and in adults. Among them was an unusual splice variant of Dβ1, where a splice site upstream of Dβ1 is spliced to the beginning of the Cβ sequence.

The primers employed in this screen were designed to amplify V regions and thus full VDJ rearrangements. It is fortuitous that they also amplify these transcripts. It is very possible that many other JnC splice products are being produced from this sterile transcript but are not detected by these primers. Although this PCR protocol is not designed to be strictly quantitative, it is clear by the number of amplification cycles and the nesting required, that this is not an abundant transcript in this RACE library. Because a mixture of products were amplified, and because the internal Cβ control gave a strong band, this most likely represents the start of sterile transcription from the *Xenopus* TCRβ locus. Full rearrangements were never detected in tadpoles of 0, 1, 2 or 4 dpf (see table 4.2). J-C and D-J-C transcripts are detected in RACE libraries of 23 dpf tadpoles.

4.6.5 Multiplex PCR

The quality of cDNA was tested by amplification with either RAG1 or EF-α primers. The presence of Cβ region transcription or possible rearrangements was assayed by internal amplification of Cβ. This is not a very good marker of T cells, because other cell types in mice and humans produce sterile Cβ transcripts [60-62], but it is an excellent positive control where the use of plasmid or cDNA from older tadpoles could lead to cross-contamination of samples. A multiplex PCR approach was taken (figure 4.7).

The TCR Vβ primers used in this study (table 4.1) were tested on adult or late tadpole cDNA and gave consistent results at the conditions outlined in the materials and methods. PCR products were confirmed by sequencing and hybridization. After this bands of the proper size were considered positive results. Bands from the day 7 and earlier were always sequenced.

All Vβ segments can be used during both tadpole and adult life (figure 4.8). Figure 4.9 shows a small sample of typical muliplex PCR experiments. The results of these studies are compiled in table 4.2.

4.6.6 The first TCR β rearrangements are detected day 6 post-fertilization

The first fully rearranged TCR β chains were found on day 6 in both the thymus and the body (liver, gut stomach) (figure 4.9). In day 6 thymus two rearrangements utilizing BV6s2, as well rearrangements of BV's 1, 3 and 5 were amplified. However, this represents PCR results from more than 15 cDNA samples, each consisting of RNA pooled from 10 animals. In individual experiments, a maximum of 2 rearrangements were found and many samples were negative. To ensure that this was not just a limitation of the multiplex PCR approach, a day 6 RACE library was produced. PCR amplification of this library using a primer in the adaptor paired with a Cβ consensus primer gave an incredibly high background amplification of bands other than TCR suggesting that full rearrangements are rare or non-existent in this library. Therefore, this most likely represents the onset of full TCR β rearrangements in tadpoles. Four of these five rearrangements used the *X. gilli* constant region C_G (see chapter 2). On day 7, the majority of Vβ segments can be used, but in individual samples there again appears to be some limitations in the number of segments which are used. On day 9, multiple rearrangements of 8 of 8 assayed Vβ families are detected in the thymus and liver suggesting that process of TCR β rearrangment in thymocytes is well underway. By day 15, most Vβ segments can be amplified from cDNA samples consisting of 10 pooled animals, again suggesting that even more rearranged TCR β chains are present in the sample. By day 24, all tested Vβ segments can be found in the thymus and spleen. These are the samples that are used to construct 5' RACE libraries for the repertoire study presented in chapter 5.

The vast majority (>90%) of TCR β rearrangements we sequenced are in frame. This in contrast to the situation in trout and axolotl where up to 30% of transcripts are non-productively rearranged [63]. RT PCR on day 2, 4, and 5 tadpoles has repeatedly yielded no TCRβ rearrangements. Table 4.2 contains a summary of all of our PCR data to date. The table does not represent Vβs which were found consistently in all samples but rather illustrates that, like mammals, all known Vβ segments can be rearranged during tadpole life [25, 51]. Rearrangements utilizing Jβs 3, 4, 5 and 8 are most often encountered, but show no clear age or tissue bias. The limited repertoire used in individual cDNA samples from very young tadpoles most likely represents the limited number of T cells in these animals.

4.6.7 Tadpole CDR3 junctions are shorter than adult junctions

Much of the diversity of TCRβ chains is generated at the CDR3 where V, D, and J segments are joined Nuclease activity can shorten segment ends and the activity of TdT, a non-template driven DNA polymerase, can add N nucleotides to segment ends in adults. Previous studies [45, 64] have shown that TdT appears to not be expressed in tadpoles, and our results seem to confirm this. The mean amino acid length of tadpole CDR3 is between 7.1 and 7.9 whereas in adults the CDR3 length averages 9.1 amino acids (table 4.3).

4.6.8 Class Ia transcription is detected as early as day 5 post-fertilization

The ontogeny of class Ia transcription has not previously been studied in young tadpoles. Tadpoles differ from adults in terms of class Ia surface expression, and this could affect the selected TCR repertoire. Therefore we wanted to know if class Ia mRNA was present in the animals used for the repertoire study in chapter 5.

Class I MHC sequences failed to be amplified from cDNA derived from fertilized eggs and day 2 tadpoles (figure 4.10). However, in day 5, 6, 15, 23 and 24 tadpoles a ~550 bp band was amplified and confirmed to be a classical class I sequence. The sequence corresponds a class I allele described by Shum et al [65]. Predictably, bands of the same size were amplified from LG15 RACE libraries. Water controls were negative, and no bands were amplified from samples of genomic DNA. Therefore, the tadpole bands cannot be explained by contaminating genomic DNA in the cDNA samples.

Class Ia transcription was also detected in the *Xenopus* B3B7 thymoma cell line. This result is not due to contaminating genomic DNA, because a cDNA sample to which reverse transcriptase was not added, did not produce a band (figure 4.10).

4.7 Discussion

Full rearrangements at the TCR β locus are first found 6 dpf in *Xenopus* when there are less than 100 lymphocytes in the thymus. This coincides with the first observation of CD3ε+ cells by immunohistology in the thymus, liver, esophagus and stomach, and rarely, in the splanchnopleura. By day 7 CD3ε$^+$ cells are also found in the larval skin, splanchnopluera and the lining of the gut. This occurs well before the first full rearrangements in chicken and mouse are found on embryonic day 14, respectively [22, 66]. In striking contrast, the first TCR rearrangements are detected in urodeles around 10 weeks post-fertilization [5]. Although, like chickens and mammals, *Xenopus* tadpoles are able to use the full range of TCR segments found in the adult, there appears to be a limitation imposed by the small number of T cells in the tadpole. A similar limitation in the number of B cells in larval *Xenopus* has been reported [45]. Thus, the size of the T cell compartment and not the available amount of genomic diversity seems sufficient to explain the less robust immune responses of tadpoles when compared to adults [67, 68]. The number of T cells also seems to increase faster than that of B cells, which shows clearly why MLR activity precedes humoral responses early in ontogeny [45].

4.7.1 Are the first CD3ε$^+$ cells in tadpoles γδ T cells?

The CD3ε$^+$ polyclonal serum is expected to detect not only αβ T cells but also γδ T cells. Therefore, the CD3ε$^+$ cells in the stomach and liver of day 5 and 6 tadpoles may be γδ cells or alternativly T cell precursors which are homing to the thymus. However, the CD3ε$^+$ cells in the liver and stomach stain much more brightly with CD3ε than do thymocytes at the same stage. This, and the movement of a CD3ε cell population into the skin by 7 dpf, strongly suggest that γδ T cells are present in tadpoles. In mammals and birds, the first wave of T cells to enter the periphery consists of γδ T cells with specific canonical rearrangements (reviewed in [51]). If the CD3ε$^+$ cells which move into the *Xenopus* skin are γδ T cells, the progression of T cell expression during ontogeny may be conserved from amphibians to mammals.

4.7.2 Are there NK cells in tadpoles?

CD3ε protein is present not only on the surface of T cells but also cytoplasmically in chicken NK cells, activated adult NK cells and most fetal human NK cells [57, 58]. It is therefore possible these early CD3ε$^+$ cells could represent NK cells. Adult *Xenopus* possess cells with an NK phenotype but such cells were not found in tadpoles [69]. The lack of NK activity in tadpoles which lack MHC class I expression is not unexpected. NK cells derived from β_2m or TAP deficient mice are tolerant to β_2m deficient bone marrow grafts suggesting there is a mechanism for inducing tolerance in NK cells when there is little or no expression of MHC class I on self cells [70-72]. *Xenopus* tadpoles may therefore possess NK cells that are tolerant but still CD3ε$^+$. Further studies employing thymectomized animals, which are thought to be rich in NK cells due to the missing T cells [83], may help clarify this issue.

4.7.3 Extrathymic development of T cells?

At present it is not clear if the early TCR rearrangements found in the liver represent an extrathymic population of T cells rearranging and residing in the liver or are merely T cell precursors in the process of homing to the thymus which have begun to rearrange the β locus. TCRβ D-J rearrangement in lymphoid progenitors before they reach the thymus has been documented in mice [23] and Rainbow trout [2]. We would argue that this population is more likely to be an extrathymic population based upon the results of [73] who found rearranged TCRβ chain mRNA in the intestine, liver and spleen of nine adult *Xenopus* which had been thymectomized at days 4 to 8. Because of the low numbers and the lack of clonal expansion of such cells, the authors concluded that the extrathymic pathway for T cell development is minimal in *Xenopus*. Nevertheless, a study of this population of cells in an ectothermic vertebrate may shed some light on the evolution and function of similar small populations of T cells that develop independently of a thymus in birds and mammals. Early CD3ε$^+$ cells found in the liver by immunofluorescence most likely represent a T cell subset.

4.7.5 Transcription from the TCRβ locus

Sterile (incomplete and untranslated) transcripts from the TCRβ locus are also detected in mice and humans. In mice, these transcripts are found in T cells even before the differentiation of the thymus and this transcription is independent of RAG 1 and 2 expression [74]. After RAG expression, sterile transcripts of three types are observed: i) 5' UTR constant region; ii) 5' UTR J-C and; iii) 5' UTR D-J-C. All three are detected in α β, γδ and NK T cell populations [60, 62]. In mice, there are promoters 5' of the arrays of J segments, 5' of Dβ1 and 5' of the constant regions. Transcription from the Dβ1 promoter is dependent on the presence of the TCRβ enhancer, located 3' of Cβ2 [62, 75]. All of these classes of transcripts have been detected in *Xenopus* (see figure 4.6). Thus the location of the promoters in the TCR β locus seems to be conserved in *Xenopus*.

4.7.6 CDR3 lengths are very similar to those of mammals

The length of the CDR3 junctions in adult *Xenopus* are very similar to that of mammals. Not unexpectedly, tadpole junctions were shorter, on average, than adult junctions, presumably due to the lack of TdT expression and activity in larval *Xenopus* [64]. This is also the case in B cells at the Ig CDR3 [45, 67]. One could also argue that the CTL and helper subsets show CDR3 length biases. The adult/tadpole differences in CDR3 length might then only reflect the lack of MHC class I expression and the different selection/ratio of the CD4$^+$ (class II restricted) and CD8$^+$ (class I restricted) subsets. Tadpoles are expected to possess little or no CD8+ T cells due to the lack of class I MHC. However, a comparison of CDRs from human and murine CD4 and CD8 cells found no significant differences in CDR length [76-78] nor were differences found in mice deficient in class I MHC [79]. Significant differences in the CDR lengths of CD4 and CD8 populations between adults and cord blood were found [79]. Because no significant differences were found in exonucleolytic removal or N region addition, the population shift in CDR3 length was concluded to be due to antigen exposure.

4.7.7 General Conclusions

It is not our aim to make any claims about the abundance of particular transcripts based on these studies, but rather to provide a general picture of the diversity of the early TCR β repertoire and to determine when rearrangements commence. The early repertoire is quite variable in that all Vβ segments can be used in tadpoles. Transcription of the β locus is detected by 3.5 dpf and full rearrangements are found on day 6 dpf. An added benefit of this work is the observation of CD3ε$^+$ cells entering the skin on day 7. These presumably correspond to the γδ T cells found in other vertebrates and these cells may play a major role in tadpole immunity. The tadpole repertoire therefore seems more restricted by T and B cell numbers than by a need for "canonical" αβ TCRs which would play a specific role early in ontogeny. A more detailed study of the proportional usage of TCR segments (both αβ and γδ) during development will be needed to detect any subtle differences in the repertoire. In chapter 5, we present a detailed study of the TCR β repertoire in the thymus and spleen of both tadpoles and adults.

No Text.

No Text.

BV1*	23472	GTTCTGCCGCCCTAATGAGATG	S
BV2	16228	CAGGTCTCTGGTGGT	S
BV3	16222	TGACGGTGAATCCTGGAGAC	S
BV4	23366	CGGTGACACAAAGCGAGAAAC	S
BV5	23467	TGCAGAACTTTACTGTGAAC	S
BV6*	15342	GTCAGCTGACATTCCTTGTAATC	S
BV7	23368	CATGTCAGAGTAATATGGAGAG	S
BV8*	23470	CAACAGAAACTGGGCCAAGG	S
BV9	23385	ATGTGACTGTACAGTGTGAGC	S
BV10	23371	CAGGCTGGGAGCCGAGTGTC	S
BV11	23465	GCTGAAGCTCATGATGCTCTCG	S
BV12	21155	GCTGCTGTTGGTACTGGTG	S
BV13	21158	AACTGCACAGTAGATGGAGC	S
BV14	23408	CGTGTCTCTGAATTGCTCAGTTG	S
BV15	22632	CATGATGCCACTGACTATCTC	S
BV16	22634	GGTGCAAACATAATGACAGCTC	S
BV17	22655	GACTGGTTCTGTCTCGTCC	S
BV18	22658	CGTGTGGTGCAGGTGCCATTAC	S
BV19	23463	CCGGGGACAAGGTGGAATTGAGC	S
CB(O)	16811	ATTCTTGGTGAGGCTGAGGCGGCTGC	AS
CB (I)	14995	CTTTTCCCTGACTTCCATCCCGTTT	AS
RAG1	12724	GCGCCAAGAATCTGTGTCACT	S
RAG1	12726	GTTCTGTTTCATGGTTGTCTACCA	AS
EFα	11507	GCTGGAAGCTCTTGACTGCATTCTGCC	S
EFα	11508	AACAGTCTGCCTCATGTCACGGACAGC	AS

Table 4.1 Primers used in this study

Shown are sense (S) and antisense (AS) primers used in this study. Number codes refer to codes used in the protocols presented in Appendix B.

Table 4.2 Summary of PCR experiments on the ontogeny of TCR β rearrangements in *Xenopus*

5' RACE and multiplex PCR were used to study the onset of rearrangements at the TCR β locus. Closed circles and open circles respectively show the presence or absence of a marker in at least one cDNA sample from that time point. nd stands for not done. Letter codes are as follows: h=hour, W=whole animal, H=head, B=body, T=thymus, L= liver, S=spleen and EF-a=elongation factor α. Water controls were carried out for each amplification where nesting was required. Furthermore, controls for plasmid contamination using the M13 reverse and T7 primers were routinely run (data not shown). Any bands recovered in such screens were subcloned and sequenced. Often the contaminant was not TCR but another plasmid routinely in use in the lab. Results from such experiments are included in this summary, but experiments with failed plasmid controls are excluded even where there was only a single contaminant sequence. The quality of cDNA was tested by amplification with either RAG1 or EF-α primers. The presence of C region transcription or possible rearrangements was assayed by internal amplification of Cβ (C int). This is a summary of over 100 separate experiments.

	6h	48h	60hH	60hB	66hH	66hB	72hH	72hB	84hH	84hB	d4W	d4H	d4B	d5H	d5B	d6T	d6L	d7T	d7L	d9T	d9L	d11T	d11L	d15T	d15S	d24T	d24S	AdT	AdS
1	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	●	○	●	●	●	●	●	●	●	●	●	●	●
2	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	●	●	●	●	nd	●	●	●	●	●	●
3	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	●	●	●	●	○	nd	●	●	●	●	●	●
4	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	nd	nd	nd	nd	○	nd	●	nd	●	nd	●	●
5	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	●	●	●	●	●	●	●	●	●	●	●	●
6	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	●	●	●	●	●	nd	●	●	●	●	●	●
7	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	nd	nd	nd	nd	●	nd	nd	nd	●	●	●	●
8	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	○	●	●	○	●	●	nd	●	●	●	●
9	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	●	●	●	●	●	nd	●	●	●	●	●
10	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	○	●	●	○	nd	●	●	●	●	●	●
11	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	nd	nd	●	nd	●	nd	●	●	●	●
12	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	nd	nd	●	nd	●	nd	●	●	●	nd
13	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	nd	nd	○	nd	nd	nd	●	●	●	●
14	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	nd	nd	●	nd	●	nd	●	●	●	●
15	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	nd	nd	●	nd	●	nd	●	●	●	●
16	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	nd	nd	●	nd	●	nd	●	●	●	●
17	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	nd	nd	○	nd	●	nd	●	●	●	●
18	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	nd	nd	●	nd	●	nd	●	●	●	●
19	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	nd	nd	●	nd	●	nd	●	nd	●	○
C int	○	●	●	●	○	○	●	●	●	●	●	●		●	●	●	●			nd	nd	●	nd	●	nd	●	●	●	●
C sterile										●	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd				
JC										●	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	●	○	●	●
DJC	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	●	○	●	
cryptic	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	●		●	
RAG1	○	♦				●					●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Efa	●	●	●	●	●	●	●	●	nd	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

Table 4.3 The length of the CDR3 in TCR β chains

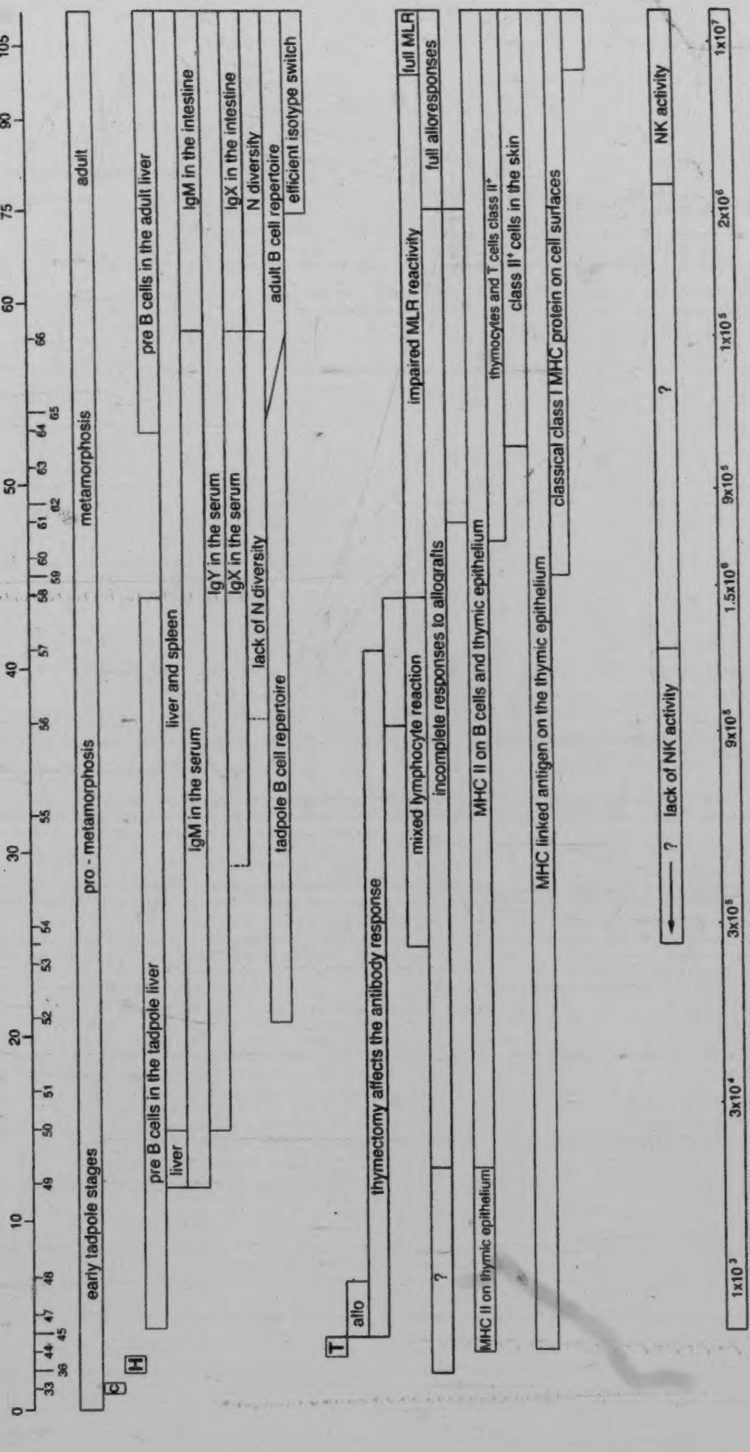
	# CDRs	Range^	Median	Mean
6 dpf	5	6,9	7	7.4
7.5 dpf	5	4,9	9	7.6
9 dpf	20	5,9	7	7.1
10 dpf	5	6,9	7	7.2
14 dpf	9	7,9	8	7.9
15 dpf	22	6,10	7	7.8
24 dpf	42	5,11	7	7.6
adult	44	6,12	9	9.1
human	97	6,12	9	9.5
mouse	147	4,13	9	8.9
sheep and cow	22	8,13	nr	10.1

Table 4.3 TCR β CDR3 lengths

CDR length is calculated as the number of amino acid between CXXX and the conserved GXG motif in the J segment minus/less four amino acids, as defined in [81]. "*"denotes the number of CDRs analyzed. ^ denotes range in amino acids as lowest value, highest value. Mammalian length values are taken from [81,82]. Ruminant median values were not reported (nr).

Figure 4.1 The ontogeny of the immune system and of immune responses in *Xenopus*.

The upper timeline shows development in days (top) or in stages (bottom) as per the Nieukoop and Faber normal table of *Xenopus* development [53]. "T" refers to the formation of the thymus, "H" for hatching, and "c" for the onset of circulation. Thymectomy during the period marked "allo" interferes with allograft responses and mixed lymphocyte reactions (MLR). The bottom bar shows the number of thymocytes present in the thymus during development. This figure is redrawn from [80].


0
10
20
30
40
50
60
75
90
105
33
36
44
45
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
early tadpole stages
pro - metamorphosis
metamorphosis
adult
C
H
pre B cells in the tadpole liver
pre B cells in the adult liver
liver
liver and spleen
IgM in the serum
IgM in the intestine
IgY in the serum
IgX in the serum
IgX in the intestine
lack of N diversity
N diversity
tadpole B cell repertoire
adult B cell repertoire
efficient isotype switch
T
allo
thymectomy affects the antibody response
mixed lymphocyte reaction
impaired MLR reactivity
full MLR
?
incomplete responses to allografts
full alloresponses
MHC II on thymic epithelium
MHC II on B cells and thymic epithelium
thymocytes and T cells class II⁺
class II⁺ cells in the skin
MHC linked antigen on the thymic epithelium
classical class I MHC protein on cell surfaces
? lack of NK activity
?
NK activity
1x10³
3x10⁴
3x10⁵
9x10⁵
1.5x10⁶
9x10⁵
1x10⁵
2x10⁶
1x10⁷

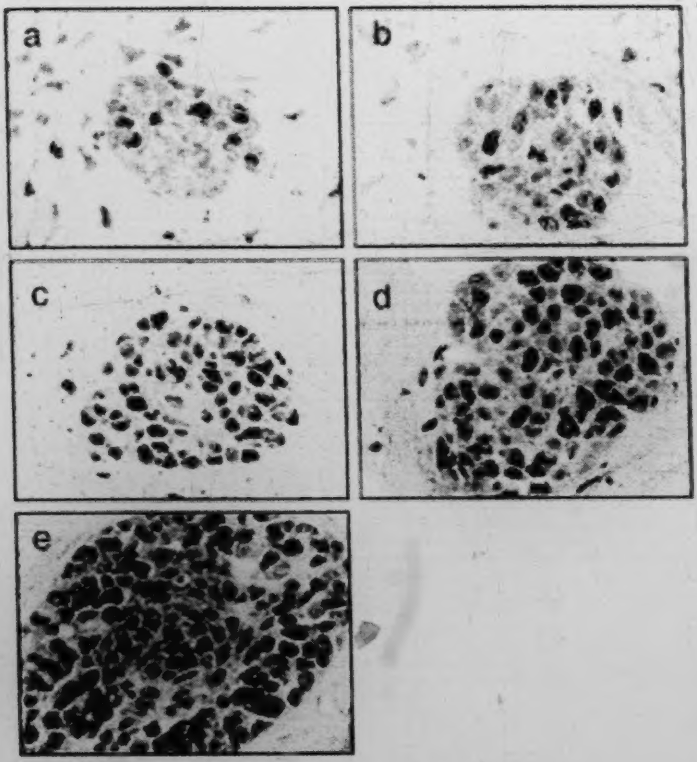


Figure 4.2 The ontogeny of the thymus

Plastic embedded sections were stained with Nocht's solution which turns metabolically active cells, in this case, lymphocytes, a deep purple colour. Shown are the thymus at (a) day 5, (b) day 6, (c) day 7, (d) day 8, and (e) day 9.

No Text

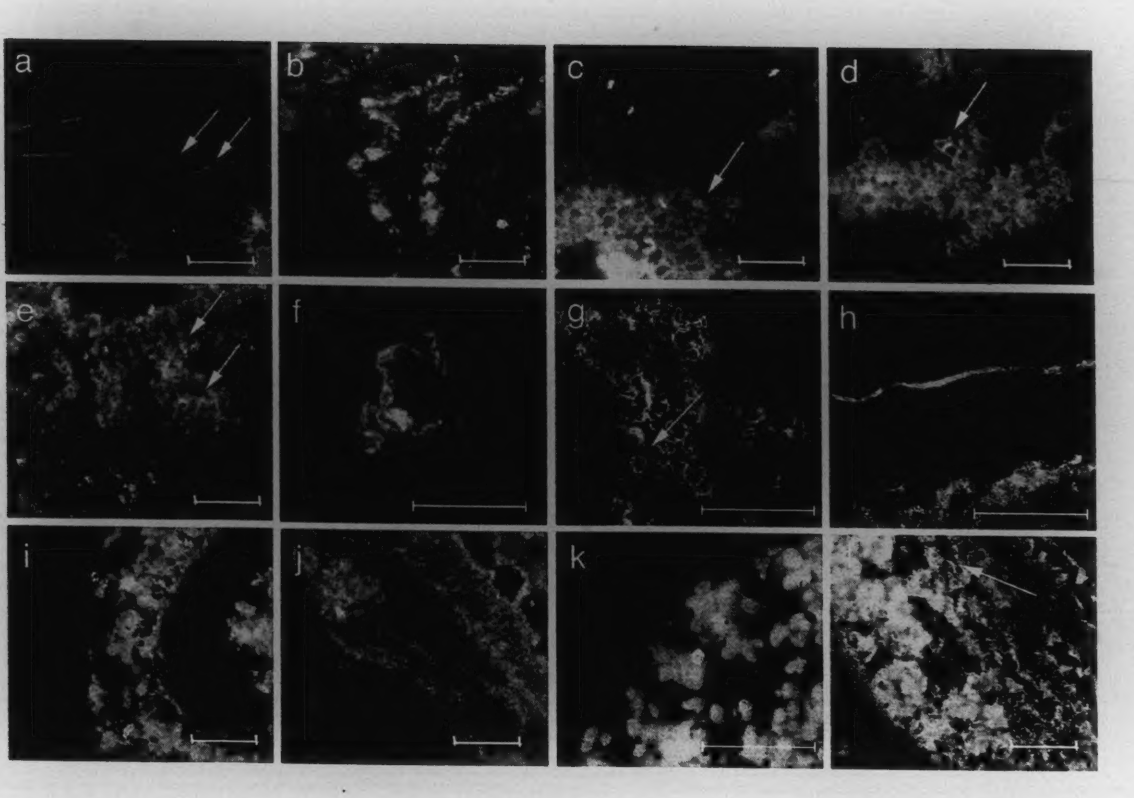
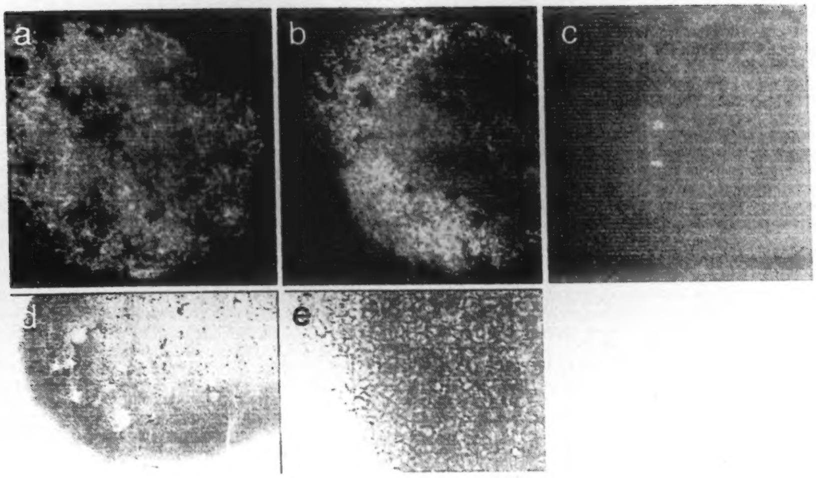


Figure 4.3 The ontogeny of CD3ε expression

Acetone fixed cryosections of tadpole tissue were stained with anti-CD3ε antiserum followed by an FITC labeled Goat anti-Rabbit Ig secondary antibody. Sections are as follows: (a) day 5 stomach, (b) day 6 esophagus, (c) day 6 gut, (d) day 6 liver, (e) day 6 stomach, (f) day 7 splanchnopleura, (g) day 7 liver, (h) day 7 skin, (i) day 9 gut with PI counterstain (in red), (j) day 9 spleen, (k) day 9 liver with PI counterstain (in red) and (l) day 9 stomach. Arrows show lightly staining positive cells. Negative controls for this secondary reagent are shown in figure 3.8. The scale bar represents 100 microns.

Figure 4.4 T cells in the larval thymus

A day 15.5 thymus was stained with the following lymphocyte specific antibodies: (a) anti-CD3ε, (b) anti-CTX [GABI] which stains cortical thymocytes, and (c) anti-B cell [10A9]. (d) and (e) show *in situ* hybridization using and antisense probe for RAG-1. Note that only the cortex is intensely stained by RAG-1 and that RAG-1 expression is not as high in the outer layers of cells of the cortex. *In situ* staining with the coresponding RAG-1 sense RNA was negative.


a
b
c
d
e

No Text

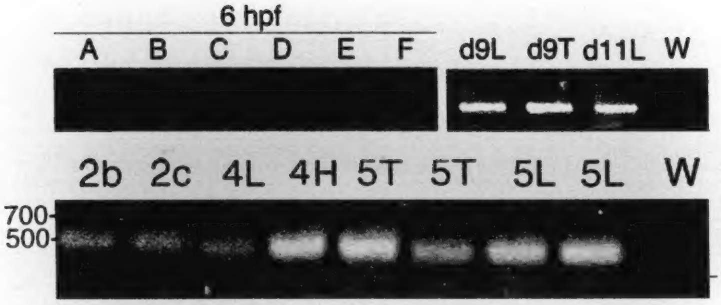

6 hpf
A
B
C
D
E
F
d9L
d9T
d11L
W
2b
2c
4L
4H
5T
5T
5L
5L
W
700
500

Figure 4.5 The ontogeny of RAG1 expression

RAG1 expression was assayed in a number of cDNA preparations. A-F represent 6 independent 6 hour post fertilization samples. Two day 2 samples, b and c, are shown as well as thymus (T) and liver (L) samples from days 4, 5, 9 and 11. These are representative of a much larger sample of RAG controls, which were carried out for cDNA samples used in this study. Water controls (W) were always carried out as well.

Figure 4.6 Sterile transcripts from the *Xenopus* TCR β locus

Several classes of sterile transcripts were obtained. These included: (a) 5' UTR C Cβ; (b) 5' UTR J-C (c) 5' UTR D-J-C and a splicing event juxtaposing an upstream portion of the 5' UTR of D-J-C transcripts and (d) the *laevis* constant region (C_L) or (e) the *gilli* constant region (C_G).

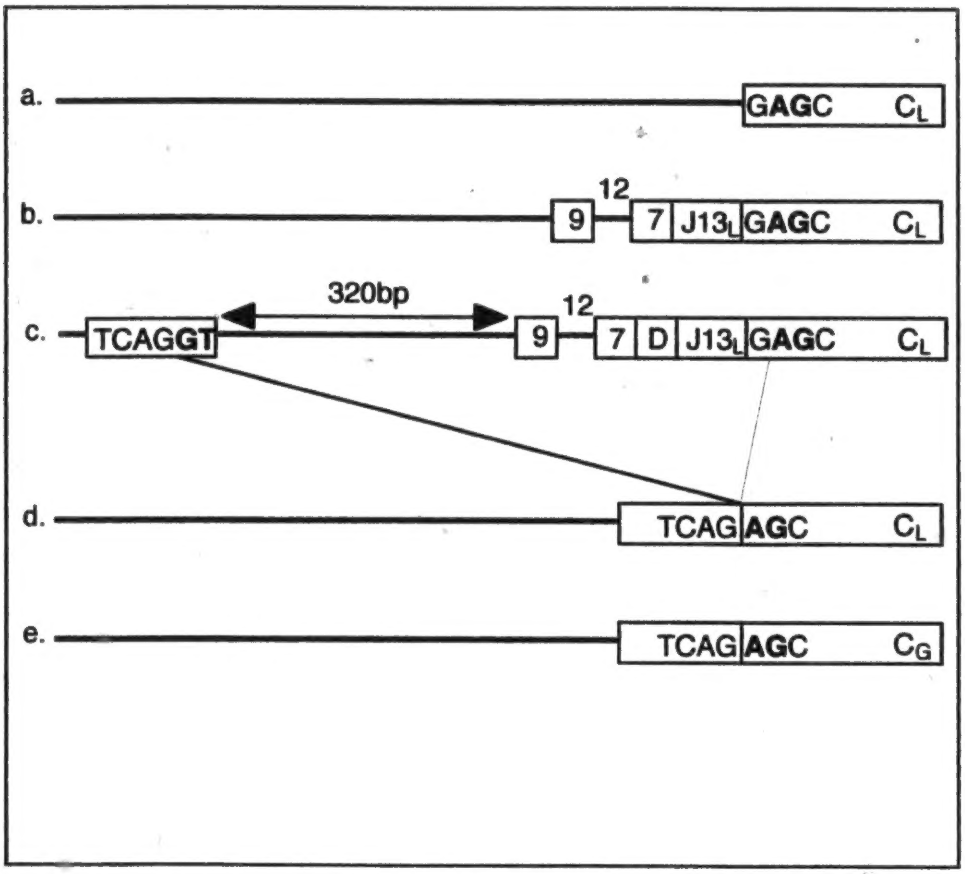
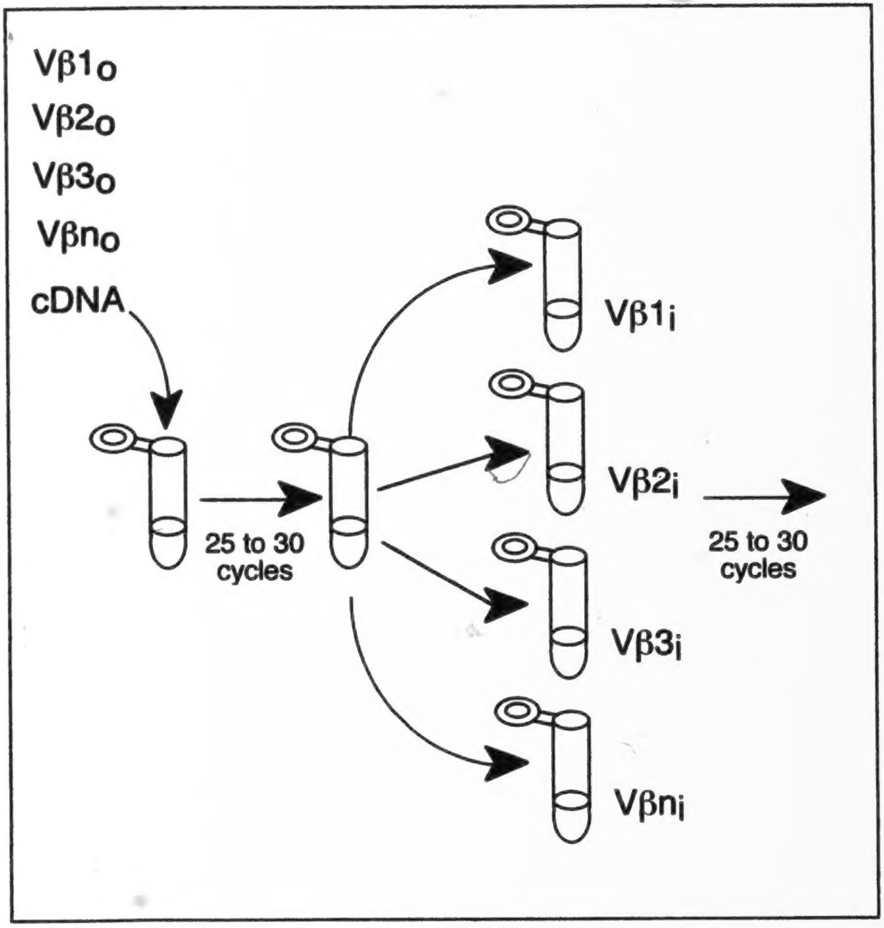

a.
GAGC
C_L
b.
9
12
7
J13_L
GAGC
C_L
c.
TCAGGT
320bp
9
12
7
D
J13_L
GAGC
C_L
d.
TCAG
AGC
C_L
e.
TCAG
AGC
C_G

Figure 4.7 Multiplex PCR

Multiplex PCR is generally used to amplify several transcripts of interest from a limiting cDNA sample. Here, a first round of PCR employs multiple outer (o) Vβ primers and a single constant region primer. After 20-25 rounds of amplification, a portion of the reaction is diluted and split into separate reaction tubes each containing a nested primer (i= inner) for a single Vβ and a constant region primer. The second round of PCR is carried out. This method can assay for the presence or absence of a particular Vβ but not the amount of transcript present.


Vβ1o
Vβ2o
Vβ3o
Vβno
cDNA
25 to 30 cycles
Vβ1i
Vβ2i
Vβ3i
Vβni
25 to 30 cycles

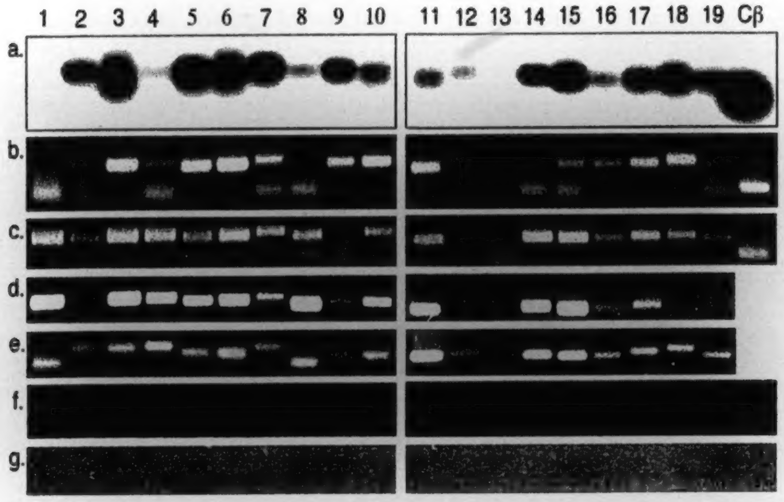


Figure 4.8 All Vβs can be used during both tadpole and adult life.

Traditional PCR with Vβ specific primers is reliable at the conditions tested and shows all Vβs can be used during both tadpole and adult life. Vβs 1 through 19 were tested as well as primers which amplify an internal Cβ fragment to test for the presence TCR transcripts in the sample (a) Hybridization of (b) using a Cβ probe, (b) and (c) represent two independent day 23 thymus samples, (d) day 23 spleen, (e) adult thymus control, (f) and (g) represent two independent day 2 cDNA samples.

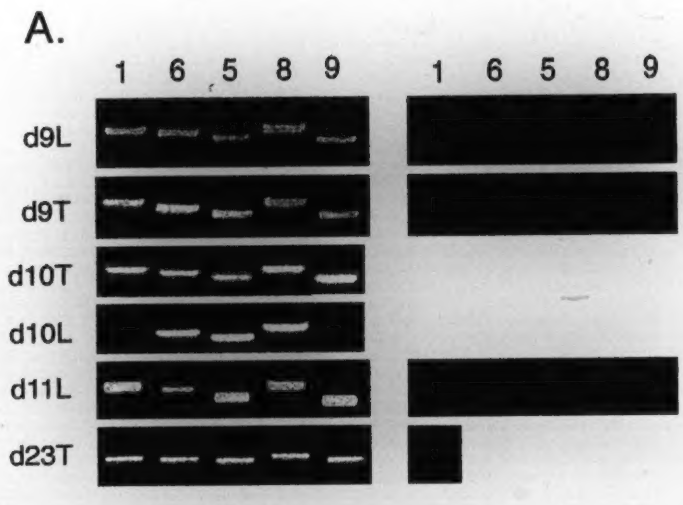
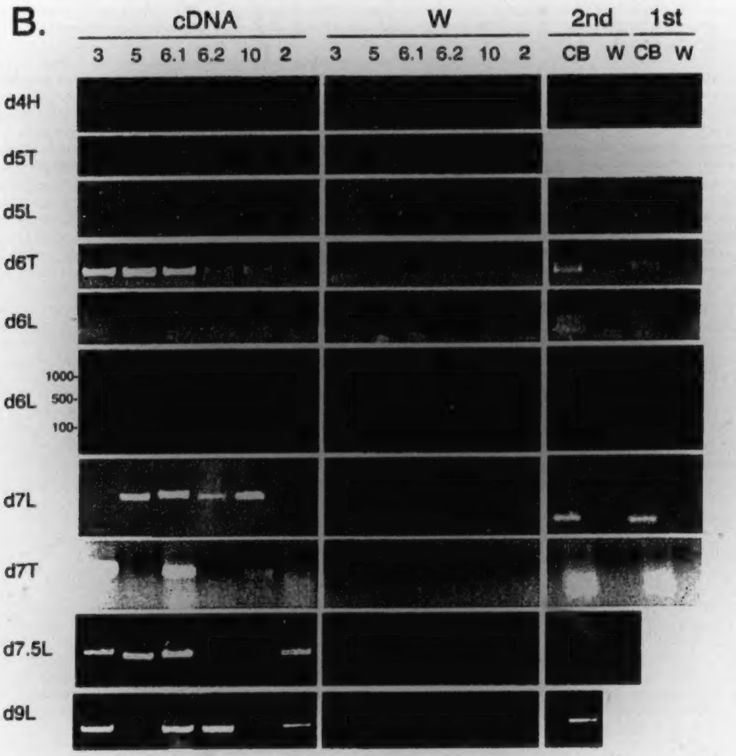


Figure 4.9 Full TCR β rearrangements are first found on day 6 Shown are sample experiments employing a multiplex PCR approach. A and B represent different mixtures of initial primers for the Vβ segments shown along the top of the panel. In A and B, the first vertical panel on the left shows the results for the templates described on the left. Letter codes are as follows: d= day; H=head; L=liver and T=thymus. The middle vertical panel contains water controls for the second amplification. The third panel, shown only for B, shows water controls (W) for the first amplification where available and internal Cβ (CB) amplifications were carried out as positive controls. The day 7.5 sample is atypical in that no internal CB band was amplified. This was most likely a problem with only that reaction, for the day 9 liver sample below was carried out with the same PCR master mix. This is only a typical sample of the type of results that contribute to table 4.2.


B.
cDNA
W
2nd
1st
3
5
6.1
6.2
10
2
3
5
6.1
6.2
10
2
CB
W
CB
W
d4H
d5T
d5L
d6T
d6L
1000-
500-
100-
d6L
d7L
d7T
d7.5L
d9L

No Text

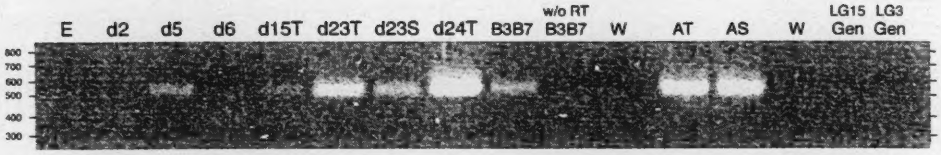

E
d2
d5
d6
d15T
d23T
d23S
d24T
B3B7
w/o RT B3B7
W
AT
AS
W
LG15 Gen
LG3 Gen
800
700
600
500
400
300

Figure 4.10 Detection of classical class I MHC transcripts in tadpoles and B3B7 by RT PCR.
Primers in the α1 and α2 domain *Xenopus* MHC class Ia were used to amplify class I message from the templates shown. Samples are as follows: E= egg cDNA, d=day, T= thymus, S=spleen, B3B7=the *Xenopus* B3B7 thymoma cell line; W=water, AT= adult thymus and AS= adult spleen. A mock cDNA synthesis was carried out without reverse transcriptase (w/o RT B3B7) to test if genomic DNA contamination of the sample could account for the band amplified from B3B7 cDNA. As well, genomic DNA controls from both LG3 and LG15 frog lines (which differ at their MHC loci) were negative. The amplified band from day 5 tadpole was subcloned and sequenced and found to be a class Ia sequence.

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Chapter 5

Expressed Thymic and Peripheral Repertoires During the Development of Two Lines of *Xenopus* Hybrids

5.1 General introduction

The finding that genes encoding the T cell receptor are produced by somatic recombination of many variable gene segments sparked a great interest in the proportional usage of these segments during the formation of the "repertoire" of T cells in an individual. Were TCR segments used randomly during recombination or were there canonical programmed rearrangements? Could all TCR segments be used in the periphery or were only some able to interact with MHC molecules during positive and negative selection? Were certain segments associated with individuals possessing a particular MHC allele? Could all TCRs interact with both class I and II MHC molecules or only with one type of MHC? These questions have been addressed in over 3000 published papers detailing the variability, selection and specificity of TCR repertoires in normal, genetically manipulated and afflicted animals. However, all of these studies except for one, have been performed in chickens and eutherian mammals, generally of medical or commercial interest. What would be the general comparative value of studies done in an externally limited sample of vertebrates? A recent study has examined the variability of the expressed TCR β repertoire in trout. This study concluded that the repertoire contained a great diversity of different TCRs but did not address the proportional usage of TCR segments within this population.

The work presented in this chapter represents the first detailed study of the expressed TCR repertoire in a poikilothermic animal. The normal proportional usage of 18 of 19 known *Xenopus* Vβ families is determined for the thymus and spleen of both tadpoles and adults. The thymic (unselected) and splenic (selected) repertoires are compared to determine the effect T cell selection on the repertoire and the repertoires of tadpoles and adults differ. Repertoire selection in tadpoles is expected to differ from that of adults due to the lack of class I MHC expression, and therefore lack of selection of CD8 T cells on class I MHC molecules in tadpoles. Does this lack of MHC expression in tadpoles result in shift in the T cell repertoire due to the presence or absence of a specific population of T cells in the tadpole? By comparing the repertoires in the two lines we hope to differentiate changes in repertoire caused by developmental differences of MHC class I expression with those due to T cell selection on differing MHC alleles. As a backdrop, we shall consider how MHC influences repertoires in mammals where the question has been addressed experimentally by opposition to the tadpole where it is addressed naturally.

5.1.1 How is repertoire measured?

Let us start with a discussion about how repertoires are measured. First, early studies of T cell receptor repertoires employed molecular techniques to quantify TCR transcripts from a population of T cells (as reviewed in [1]). Most of these studies employed polymerase chain reaction (PCR) based protocols. The least reproducible of the PCR methods is amplification using V region specific primers [2]. The priming of different V region primers is seldom equally efficient and therefore attempts to quantify and compare Vβ expression in this manner are often very biased [85, 86].

More reproducible is anchored PCR [2], sometimes referred to as rapid amplification of cDNA ends (RACE) PCR. In anchored PCR an adapter sequence is ligated to the 5' end of all molecules in a cDNA sample [3-6]. Amplification with a primer in the adaptor and in the constant regions allows an amplification of all TCR sequences in that sample. This technique is quantitative if the PCR amplification is not excessive. A drawback of this method is that it is not well suited to measure small T cell populations. The blunt end ligation used to add the adaptor is not very efficient and a significant portion of the repertoire does not gain an adaptor. Subsequent amplification leads to an extremely biased repertoire.

Immunoscope analysis has also proven extremely useful [7-14]. This technique involves amplification of TCRβ sequences using specific Vβ and Cβ primers. The products amplified in this initial PCR reaction are then digested with a restriction enzyme specific for a site in the Vβ. Fluorescent labelled Cβ primers are used to prime run-off reactions resulting in labelled fragments which vary in length depending on the length of the CDR3 region of each template. This population of fragments is then separated on an ABI polyacrylamide sequencing gel and peaks of fluorescence are seen for each CDR3 length in the population. The peaks are separated by 3 bp, consistent with a population of in frame VDJ rearrangements. A normal diverse TCR repertoire will give a set of 5-8 peaks with a Gaussian distribution. Responses to viral infections lead to a proliferation of individual T cell clones and skew the immunoscope results to only a few predominant bands. Immunoscope spectrotyping is an excellent method for determining the diversity of sequences that use a specific Vβ segment, and is the most viable way of quickly determining if the repertoire has been skewed by infection. Infection can "skew" the repertoire by leading to a proliferation of a few T cells. All of these T cells will have the same rearrangement and therefore the same length of CDR3 region. This is detected by the immunoscope as a few predominant peaks rather than the normal Gaussian distribution. However, because the initial amplification is carried out with distinct Vβ specific primers, the proportional usage cannot be reliably determined in this manner.

Many more recent T cell receptor repertoire studies, in mice and humans, have employed monoclonal antibodies raised to specific V regions [15-17]. This technique allows large numbers of cells to be characterized using fluorescence activated cell sorting (FACs). Although fast, the major drawback of this method is the limited availability of V specific antibodies in mice and humans. Repertoire studies in other vertebrates are limited to molecular techniques due to the unavailability of TCR specific antibodies.

5.2 A review of TCRβ repertoire in mice and humans

The vast majority of studies on TCR repertoire have been carried out in mice and humans. From the time it was realized that the TCR interacted with peptide-MHC complexes there has been intense interest in the TCR repertoires selected on different MHC backgrounds and if specific TCR segments are associated with certain MHC alleles (reviewed in [2, 18]). Considering the huge body of work on this topic, only those aspects, which have a bearing on the *Xenopus* study presented in this chapter, will be reviewed here. This includes a reminder of the normal fetal and adult repertoires of mice and humans, which was discussed in chapter 4, section 4.3.1. In addition, because *Xenopus* tadpoles do not express class I MHC, the TCR β repertoire in class I deficient mice and the residual development of CD8 T cells in these murine systems is reviewed. Finally, *Xenopus* tadpoles and adults should differ in the proportion of CD8 to CD4 T cells (note: this is implied due to the need for a reagent that recognizes CD4 in *Xenopus*) because of a lack of class I MHC in tadpoles upon which to select CD8 T cells. Thus, repertoire differences between CD4 and CD8 T cells in mice and humans are discussed.

5.2.1 The normal TCR β repertoire in mice

VDJ rearrangement at the TCR β locus is not a fully random process. Several Vβ segments are preferentially associated with specific Jβ segments, but it is not clear if this represents an inherent bias in recombination or the secondary effects of selection [19]. Some recent studies have shown some of these biases are due to recombination or to positive selection. There are also reproducible repertoire differences seen between inbred mouse strains, and the repertoire of mice from the wild is much more variable [20]. Thus, the repertoire is at least partially genetically influenced.

The Vβ and Jβ usage of T cells does not vary significantly between fetal and adult mice [2]. However, unlike fetal cells, adult T cells express TdT, which allows the addition of nucleotides at V-D-J junctions thus increasing the available repertoire in adults [21, 22]. A lack of TdT during embryonic life is also observed in skate, some fish, amphibians, birds and mammals [23-27] as reviewed in [28] and [29]).

Casrouge et al, [30] have estimated the size of the mouse repertoire based on an extensive sampling of the splenic repertoire. They conclude that there are 1-2 $x10^6$ different receptors in 2×10^7 cells. Thus, there are approximately 10 cells per receptor specificity in the mouse peripheral repertoire. This is significantly lower than the number of receptors predicted by calculating random usage of TCR segments $1x10^{15}$ [18]. However, a mouse has roughly $1x10^8$ T cells and only $1x10^7$ splenocytes to carry this diversity [30].

5.2.2 The normal human repertoire

By the end of the second trimester, human fetal thymocytes use the entire repertoire of 20 Vβ families [31]. Within fetal individuals, the proportion of usage of Vβ segments in the thymus and spleen is very similar [31]. This, perhaps surprising, lack of a selection imprint on the peripheral repertoire may be due to the relatively antigen free environment provided in the womb or may represent a higher proportion of mRNA in thymocytes being derived from selected thymocytes [31]. Alternatively, it could be a general feature of selection and repertoires, as a similar pattern occurs in the thymic and peripheral repertoires of adult mice [31, 32]. Some repertoire differences in human CDR3 lengths were found in between fetal (cord blood) and adult but not in between CD4 and CD8 subsets [33]. This group claims that these differences are not due to developmental differences in TdT expression but rather reflect a shift in the population due to antigen exposure. Such differences are unlikely to bias studies of *Xenopus* repertoire for the results presented in this chapter are collected from animals less than a year and a half old. The usage of TCR (segments in the fetal and adult repertoires in mice and humans are very similar, showing no reproducible significant differences [2, 34]. All TCRβ segments can be used by fetal or adult T cells.

The size of the theoretical repertoire has been a point of contention for many years. Assuming the random usage of all segments and junctional diversity at the joins as well as the efficient pairing, and surface expression of all α with all β chains, one estimates the diversity of the TCR αβ repertoire to be 10^{14} different receptors [35]. A more recent estimate based on a sampling of the actual human repertoire led to an estimate of 2.5 x 10^5 and 9 x 10^5 α and β chains respectively, thus leading to a maximum diversity of 10^8 different receptors [36]. Borghans suggests that this estimate is too low because a single β chain can bind to multiple α chains and after β rearrangement the pre-T cell undergoes a 1000 fold proliferation [37]. This allows a single TCR β chain to interact with multiple α chains. Taking this into account, the thymic repertoire is estimated at 10^{11} different receptors.

5.2.3 Mice with genetically modified class I MHC expression

Tadpoles do not express classical or non-classical MHC until just before metamorphosis. Several murine models address the role of class I MHC in the selection of $CD8^+$ CTLs. These models involve disturbing or eliminating class I expression by interfering with the transport of peptides needed to stabilize the MHC I heterodimer (TAP) or by eliminating the β_2m subunit of the heterodimer. Although TCR repertoires are not available for all of these models, tadpoles may have similar modulations in their $CD8^+$ T cell populations and thus these models are of interest.

5.2.3.1 $TAP^{-/-}$

The transporter associated with antigen processing, (TAP) is made up of two subunits and is necessary for transporting peptides from the cytoplasm into the lumen of the ER where they are loaded onto class I molecules [38]. Class Ia molecules are unstable without bound peptide. Nevertheless, mice deficient in TAP are not fully class I deficient because small numbers of class I heterodimers continue to reach the cell surface and can be stabilized by exogenous peptides [39]. These mice have significantly higher number of $CD8^+$ T cells in the periphery than $\beta_2m^{-/-}$ mice [40] (described below).

Although the size of the CD8$^+$ T cell population is limited in TAP2 $^{-/-}$ mice, they show a diverse repertoire of TCR Vβ segments. Vβ usage is very similar to that of wild-type mice except for significant decreases in the usage of Vβ 5.1 and 5.2 [41].

5.2.3.2 β_2m $^{-/-}$ mice

β_2m is a vital part of the classical MHC class Ia heterodimer and also for expression of some non-classical class I MHC molecules such as CD1. Therefore, β_2m disruption greatly reduces surface class I expression, however, residual class Ia surface expression is detected in this system, resulting in the selection of functional CD8$^+$ T cells in the periphery (as reviewed by [42]). Despite greatly reduced numbers of CD8$^+$ cells, β_2m deficient mice possess a fairly normal peripheral CD8 repertoire but show a reduced usage of Vβ5 and Vβ6 [43]. Delayed tumour rejection is associated with these mice but they are able to reject MHC disparate skin grafts [44, 45]. β_2m$^{-/-}$ mice also possess functional NK cells which are inactive or tolerant in β_2m$^{-/-}$ animals [46, 47].

This is an attractive model from a *Xenopus* viewpoint, for it describes a situation where class I expression is hard to detect and there is a low number of CD8$^+$ T cells in the periphery, as is the situation in the tadpole.

5.2.3.3 TAP1$^{-/-}$/ β_2m $^{-/-}$ double knockout mice

Although it is generally thought that β_2m and TAP1 are absolutely necessary for surface expression of class I molecules, some heavy chains do make it to the surface of thymus epithelial cells and are able to select a very small, but functional CD8$^+$ population. However, although MHC class I expression is not detectable by immunoprecipitation or immunofluorescence, alloreactive CTLs from wild-type mice on the same background made vigorous responses thus showing a very low but functionally significant expression of surface MHC in these double knockout mice [48].

It is important to remember that class I expression is not only important for T cell selection but also for NK cell function. NK cells generally attack cells lacking MHC class I expression, however, NK cells in TAP1$^{-/-}$/ β_2m $^{-/-}$ mice are tolerant to class I negative cells.

This tolerance can be broken by stimulating these NK cells with the cytokine IL-2 for 4 days *in vitro* [49, 50]. Therefore, these cells are functional but anergic suggesting that there are mechanisms for inducing tolerance in the NK population.

Knocking out the function of class I MHC by interfering with β_2m or TAP expression was not efficient enough to determine the effects of a true lack of class I expression on T cell development and repertoire. For this reason, mice with disrupted classical class I heavy chains (H-2K^b and H-2D^b) were generated [51].

5.2.3.4 H-2 $K^{b-/-}$ and H-2 $D^{b\ -/-}$ single knockout mice

H-2K^b or H-2D^b were knocked out in C57BL6 mice who possess a natural deletion of H-2L^b [52]. Surprisingly, the removal of K^b results in a 30 to 50% reduction in the CD8 population while the removal of Db gives an almost normal number of CD8+ T cells in the periphery (reviewed in [53] [51]). This argues against the theory that an increase in the number of MHC loci and alleles expressed in an individual will always lead to a greater number cells being deleted on additional MHC haplotypes [54]. It is also intriguing that the two loci are not equivalent in their effect on the selecting the CD8+ compartment (see section 7.6.1).

5.2.3.5 H-2 $K^b D^{b\ -/-}$: a true knockout of the classical class I in mice

H-2 $K^b D^{b\ -/-}$ mice express no detectable classical MHC class Ia heavy chains, but do express β_2m and non-classical MHC class I [51]. Classical class I in these mice is not detected by fluorescence activated cell sorting (FACs), immunoprecipitation or immunoblots.

There is a large (>90%) reduction in the number of $CD8^+$ cells in these animals [30]. This severity limits their ability to mount efficient primary immune responses to foreign cells (alloresponses). Only if animals are immunized with foreign cells (*in vivo* priming) does one see strong alloresponses [51]. This may be very similar to the tadpole situation where there are limitations in the number of $CD8^+$ cells.

However, the selected peripheral repertoire of $CD8^+$ T cells can recognize antigen presented by both class Ia and b molecules and mount a protective response to *Listeria monocytogenes* [53]. The decrease in the number of peripheral CD8- cells in these mice is compensated by an increase in the number of cells in the CD4 population. The decrease in T cell number is similar to that of $\beta_2m^{-/-}$ animals [51, 53]. NK cells are also present in these animals but, like other mice with low amount of surface MHC, are somehow made tolerant [51].

Classical class I MHC molecules are responsible for the selection of most $CD8^+$ cells, but the residual $CD8^+$ population in the double heavy chain knockouts suggested that $CD8^+$ T cells might also be selected on class Ib (non-classical) molecules.

5.2.3.6 H-2 K^bD^b $\beta_2m^{-/-}$

Several non-classical MHC class I molecules are dependent in β_2m for their expression. When the expression of these β_2m dependent class Ib molecules is eliminated by breeding classical MHC class I knockout mice onto a $\beta_2m^{-/-}$ background, no $CD8^+$ T cells can be detected [53]. This highlights the importance of non-classical MHC class I molecules in the selection of a small CD8β T cell subset. In *Xenopus*, we currently cannot rule out the possibility that a non-classical class I molecule remains to be discovered which may be responsible for selection of $CD8^+$ cells in tadpole life.

5.2.3.7 Repertoire differences between CD4 and CD8 T cells

The disruption of a class II gene in mice leads to a decrease in the number of $CD4^+$ T cells but the $CD8^+$ population increases to compensate [55]. If there are differences in the repertoire of TCRβ segments used in $CD4^+$ and $CD8^+$ cells, then repertoire differences between adults and tadpoles may reflect this. The question of $CD4^+$ and $CD8^+$ TCRβ repertoires has been well studied in mice and humans.

In mice and humans, all TCR Vβ regions are used by both class I and II restricted TCRs, but some segments are more prevalent in one subset [56-65]. A growing number of available TCR/peptide-MHC crystal structures show that the TCR/MHC contact points are very variable, defying a basic rule to explain the TCRs seeming evolution to recognize MHC molecules (reviewed in [66]). Other work has suggested that approximately one third of unselected TCRs can recognize a peptide MHC complex in the thymus [66]. Therefore TCR/MHC interactions seem to be evolutionarily selected for interactions with MHC but are subtler than previously thought.

Despite greatly reduced numbers of $CD8^+$ cells, β_2m deficient mice possess a fairly normal peripheral CD8 repertoire but show a reduced usage of Vβ5 and Vβ6 [43]. Likewise, $TAP2^{-/-}$ mice show a diverse repertoire of TCR Vβ segments. Vβ usage was very close to that of wild-type mice except for a significant decrease in the proportional usage of Vβ 5.1 and 5.2 [41]. Associations of a particular Vβ with developmental stage or a particular T cell subset seems to be an exception rather than a rule.

Recent studies using a mouse line with a transgenic (tg) TCRβ construct and a TCRα minilocus with a single Vα and two Jαs [66]. This model has provided a minimal but variable repertoire of T cells and is being used to look at how the T cell repertoire is shaped, from the unselected repertoire, through selection, and through peripheral tolerance. These "limited" mice possess a repertoire that, while still variable, shows preferential usage of some segments. This appears to be imposed already at the level of positive selection. Thus, a subset of VαJαVβJβ combinations more likely than others to be selected on self-peptide-MHC complexes. Furthermore, although ~10% of the TCRs can interact with and be selected on both MHC classes, there is a distinct bias toward one or the other compartment. Intriguingly, when limited mice are bred onto a class II deficient background, many of the sequences previously associated with selection into the CD4 lineage are now found in the CD8 population. If this were true in the tadpole population then the tadpole may possess unique CD4 specifications that in adults would always be selected into the CTL compartment.

Furthermore, changes in all three of the TCRs CDRs can affect selection into the CD4 or CD8 compartments. It is interesting to note that small changes in CDRs 1 or 2 could bias TCRs for selection on class I or II MHC molecules. These residues are located centrally over the peptide such that the bias may be due to contacts with the peptide rather than with the α helices of the MHC, as is commonly thought [65-67]. Changes in the CDR3 regions could also bias cells into one lineage or the other thus interactions with the peptide are likely to be a very important part of MHC restriction. This is also supported by large changes in the repertoire when cells entered the periphery suggesting that the different peptide landscape in the periphery [68] plays a major role in shaping the repertoire.

5.2.4 Differences in individual repertoires

Studies in twins suggest that the TCR repertoire may be more correlated to the TCR present in an individual than the MHC loci upon which this "raw" repertoire is tested. Studied of human twins as compared to HLA matched siblings, showed that the TCR locus or differences in the peptides presented on MHC molecules has a profound effect on the TCRβ repertoire.

Intriguingly, in humans the CD4 repertoire was found to be quite similar in different individuals but the CD8 repertoire varied more widely even in identical twins presumably due to differences in antigen exposure. [58, 62]. Thus, it is possible that there is a difference in the role of the peptide in determining the repertoire in these two subsets because foreign peptides do not lead to differences in the CD4 repertoire.

This issue is particularly significant in the case of this *Xenopus* repertoire study, for LG hybrids can be gynogenetically cloned. We are, in essence, working with populations of monozygotic twins. The TCR loci in LG3 and LG15 are identical as confirmed by Southern hybridizations presented in chapter 2.

This suggests that, although the *Xenopus* animals used to study the TCR β repertoire are genetically identical (isogenic cloned lines), antigen exposure may lead to perturbations predominantly in the CD8 repertoire.

5.2.5 Superantigens

Superantigens are molecules (sometimes produced by pathogens) which activate T cell populations by cross-linking the outside surfaces of the TCR and MHC molecules [69-71]. This does not interfere with the normal TCR/ peptide-MHC interaction but it supercedes the specificity of the receptor the result being that all T cells bearing TCRs with a certain Vβ segment are activated. This can result in an activation of 15 – 20% of the total repertoire. If these superantigens are present in the thymus they result in the negative selection (sometimes called deletion) of an entire subset of T cells expressing the same Vβ segments, forming a "hole" in the repertoire. This is common in mice, but SAgs do not seem to affect the human repertoire as completely (as reviewed in [72]).

Preliminary experiments in axolotl, a urodele amphibian, have suggested that SAgs can cause an activation and proliferation of entire sets of T cells but this has not been further characterized [73].

Here we will see if such deletions exist in *Xenopus* repertoire. Results shown in figure 4.8 show all TCR β segments can be used during tadpole and adult life and suggests that major deletions will not be found. However, the H4 region of TCR Vβ segments interacts with superantigens. The H4 regions of *Xenopus* TCR Vβ segments are quite variable (see chapter 2) and this implies that SAg have acted as a selective pressure. Here, a closer look at the repertoire may show some subtler effects on TCR β repertoire.

5.2.6 Summary

Work done in mice and human TCR β repertories suggest that rearrangement and positive selection bias the repertoire leading to non-random patterns of usage during both fetal and adult life. The TCR repertoire is well correlated to the TCR loci present in the individual, suggesting that there is a strong genetic component to repertoire. This non-random repertoire, is then molded through selection on MHC molecules in the individual. Many T cell receptors can be selected on both class I and II molecules but there are few reproducible biases in the Vβ or Jβ repertories selected on class I or II MHC molecules. Superantigens produce the most profound effect on the repertoire leading to the deletion of up to 20% of the peripheral repertoire in mice. SAg have less effects on the human T cell repertoire for reasons that are still unknown.

5.3 TCR repertoires in poikilothermic animals

Studies of the expressed repertoire of TCR rearrangements in poikilothermic animals have been limited by an unavailability of TCR specific antibodies and the fact that an exhaustive cloning of TCR segments has not been carried out.

Repertoire studies have not been systematically carried out in poikilothermic vertebrates except rainbow trout where the diversity of the CDR3 regions of TCRβ chains was assayed by immunoscope [9]. In other poikilothermic species, TCR segments have often been cloned from thymus or spleen cDNA libraries. Generally quite a number of TCR β segments are recovered from a small number of clones. In *Raja eglanteria* (clearnose skate), 4 Jβ and 2 Cβ segments were found in 17 cDNAs [87]. In the *Heterodontus francisci* (horned shark), an analysis of 41 cDNAs led to the discovery of 7 Vβ, 10Jβ, 4 Cβ, and a single Dβ segment [88]. In axolotl a larger sampling was done.

Analysis of more than 250 cloned VDJ regions led to the discovery of 13 Vβ, 13 Jβ, 4 Dβ and 4 Cβ segments [89]. All of these results suggest that there is considerable diversity in the expressed repertoire of TCRβ chains in these species [90]. However, these kinds of studies do not give us much information concerning the proportional expression of segments or the number of T cell clones present in a sample. Both PCR and the process of making a cDNA library can bias the measured repertoire unless carefully controlled. Therefore the question of expressed TCR repertoire remains to be addressed in poikilothermic vertebrates.

Recently, the diversity of TCRβ expression in the thymic and peripheral repertoire was studied in trout using immunoscope analysis.

Immunoscope analysis of T cell populations in the trout thymus, spleen and pronephros (a secondary lymphoid organ in fish) showed no major differences suggesting that the trout thymic and peripheral repertoires are polyclonal and not skewed because of recombination constraints or selective pressures. This distribution changed upon viral infection of the trout, showing one or two dominant rearrangements. Therefore, the trout study can only conclude that the trout repertoire seems as diverse as a mouse or human repertoire and is similarly affected by antiviral immune responses. Therefore anchored PCR or antibody based methods, as discussed earlier, are better suited to comparative studies of Vβ usage. Spectrotyping (ie. the use of immunoscope analysis) can complement this analysis by allowing the diversity within each Vβ segments rearrangements to be determined.

The trout study is the only study to date that addresses the actual peripheral repertoire of poikilothermic vertebrates. Sequencing from cDNA libraries is not considered a study of repertoire unless precautions are taken to ensure that the sample will yield proportional results. This has not been done in studies to date so these studies are not covered here.

5.4 Variations in the T cell receptor β repertoire in *Xenopus* tadpoles and adults

5.4.1 Introduction

This study was carried out to determine (a) the diversity of the thymic and peripheral repertoires in *Xenopus* tadpoles and adults; (b) to determine if there are significant differences in these repertoires; (c) if there are such differences in the repertoires, what is the most important factor influencing the repertoire? Additionally we address the question of developmental differences in rearrangement by comparing thymic repertoires. Comparisons of the splenic (peripheral) repertoires will address if there are differences in selection in the absence of class I MHC in the tadpole when compared to that of the class I and II MHC positive adult? If there are developmental differences which we might attribute to the absence of class I in tadpoles, are the same differences found in a second frog line which possesses the same TCR loci but a different MHC haplotype? Such conserved differences suggest that the lack of class I MHC expression might ensure the presence of T cells with or without a particular Vβ segments during tadpole life.

5.4.2 Materials and Methods

5.4.2.1 Animals

Hybrid LG15 animals were produced by gynogenesis as previously described [74]. LG3 and LG15 clones are respectively of haplotypes bd and ac at the class I and MHC loci but share the two alleles of the TCR β locus [75-77]. Animals were sacrificed at 24 or 25 dpf at stage 53 [78] and thymus and spleen tissue taken. Adults, were between 6 and 7 months old when sacrificed.

5.4.2.2 RNA Isolation

RNA isolation was carried out as per protocol in chapter 2 (section 2.1.2) and in appendix A.

5.4.2.3 Smart RACE library construction

The Smart RACE Kit from CLONTECH was used as per protocol with a few minor modifications as per protocol in chapter 2 (section 2.1.2) and in appendix A.

5.4.2.4 Subcloning and screening of colonies

RACE amplification products were separated on a 1% agarose gel and bands greater than 500bp were purified from the gel. Initial experiments showed that partial DJC, JC and C transcripts were also amplified in this protocol, and effectively diluted the population of VDJC products when subcloned, because shorter fragments were subcloned more efficiently. The purified >50 bp fraction was subcloned into the pCR II vector (InVitrogen) by TA cloning, and transfected into TOP 10 bacteria (InVitrogen). Randomly selected clones were sequenced. Where the sequences obtained were diverse, the same ligation was again transfected into bacteria and plated [79].

Plate lifts were carried out according to the duplication protocol outlined in Sambrook and Maniatis [79]. Briefly: bacterial colonies were transferred to sterile reinforced nitrocellulose filters. These lifts were placed colony side up on a fresh plate and placed at 37°C for several hours. A second filter is placed on the first and the "sandwich" is incubated for ~2 hours, after which the two filters are removed together from the plate and lysed for 2 minutes on either side by floating them on lysis buffer (0.5M NaOH, 1.5M NaCl). Filters were then treated 3 times for 3 minutes with neutralization buffer (0.5M Tris HCl pH 7.5, 1.5M NaCl). After extensive washing with 2X SSC, they were dried and UV cross-linked. Four sets of duplicate filters were produced from each series of five plates.

5.4.2.5 Hybridization

Filters were pre-hybridized in 1% Blotto in Jim's Hybridization stock without dextran sulfate (see appendix A) solution for several hours, followed by ON incubation at 65°C with ^{32}P labelled Vβ specific probe encompassing at least 2/3 of the length Vβ segments in question. Controls for specificity consisted of plasmids of known sequence spotted in duplicate into a nylon filter. Cross hybridization was generally not a problem under these hybridization and washing protocols. Where cross hybridization was indicated on the control filter, all filters were rewashed and re-exposed. Films were exposed to filters for 1 –

6 days and filters were then stripped by washing at 70°C in 0.1% glycerol in 80 to 90°C water was used.

5.4.2.6 Sequencing and sequence analysis

Sequencing was carried out on an ABI sequencer using the Big Dye Terminator Kit and on a LI-COR system using fluorescently labelled primers and a thermosequenase kit using standard protocols. A local sequence database developed by Stefan Meyer was used to store and classify sequences using a BLAST algorithm.

5.4.2.7 Statistical methods

Hybridizing colonies were counted twice and tabulated. The full data set is given in appendix C. In data subsets with one missing value, the missing value was replaced with the subset sample mean. Where there were multiple missing values in for Vβ segment counts, the remainder of the segments counts for that condition were scaled to match the least complete data (i.e. where only 7 of 10 plate counts were available for a particular Vβ segment for a particular RACE library, the data for all other Vβ plate counts from that same library were scaled by multiplying by 0.7). This leads to an underestimation of the actual significance of the data because it reduces the number of colonies scored to allow comparison with smaller samples. It does however allow more samples to be compared. χ^2 analysis was carried out in a rxc contingency table or an exact rxc contingency table. The online calculators used for these calculations can be found at www.physics.csbsju.edu/stats/exact_NROW_NCOLUMN_form.html and www.physics.csbsju.edu/stats.

5.4.3 Results

5.4.3.1 RACE PCR and cloning

To study *Xenopus* TCR repertoires, RACE libraries were constructed. RACE (rapid amplification of cDNA ends) (figure 5.1) involves the addition of a known sequence to the

5' end of cDNAs. Amplification using a primer in the known 5' sequence and another in the TCR Cβ region allows the relatively unbiased amplification of all TCR β cDNAs in a sample. These amplified fragments are then subcloned and used to transform bacteria. Each resulting bacterial colony corresponds to a single TCR β cDNA.

The viability of the RACE libraries was tested by amplifying a diluted aliquot of the initial RACE PCR reaction (figure 5.2 A and B) containing a mixture of TCR β sequences, with a panel of Vβ specific primers. In each case, all or most Vβ segments are represented (figure 5.2 C and D). Next, libraries were sampled by randomly sequencing between 48 and 69 randomly selected colonies. Insufficient numbers of sequences were collected for LG3 tadpole and adult spleen libraries, and are not analyzed here further. Hybridization allowed larger numbers of colonies to be screened (figure 5.3). DNA from bacterial colonies was tranfered via alkaline lysis onto reinforced nitrocellulose filters and these filters were screened with specific probes for 18 of 19 known Vβ segments. 4-5 sets of replica filters were produced. Two RACE libraries were prepared for each condition except in the case of the LG15 tadpole thymus library where time constraints allowed only one library to be screened. Between 600 to 1300 colonies were screened per library allowing the proportional usage of each Vβ segment in the population to be calculated.

Here we compare the repertoires of day 24 tadpoles to 6-7 month old frogs. We decided to use 24 dpf tadpoles for several reasons. First, a thymus and a spleen are present and contain sufficient numbers of cells to obtain a characteristic "tadpole" repertoire. Second, surface class I expression has never been detected as early as day 24 ensuring that we can test the effects of a lack of class I expression on the tadpole repertoire. Finally, the T cell populations in the tadpoles have probably not had time to become activated and proliferate, which would bias any measurement of the repertoire. 6-7 month old adults were chosen because previous studies had shown that such animals can produce a full adult immune response [80, 81]. As well, the thymus involutes after one year, necessitating the study be done before that time point.

5.4.3.2 Frequency of Cβ usage

The *Xenopus* TCR β locus possesses only a single Cβ segment. Thus in LG hybrids, a *laevis* and a *gilli* "allele" of Cβ are present and can be differentiated by minor sequence differences (see chapter 2). VDJ rearrangement at the *Xenopus* TCR β locus appears to happen in *cis* (i.e. within the locus and not between the *laevis* and *gilli* loci present on different chromosomes) allowing us to trace which locus the rearrangement occurred on.

For this study it was important to determine if the usage of the two loci in the LG3 and LG15 hybrids is biased. One could imagine that the availability of trans-acting factors or the availability of the locus for recombination could lead to preferential rearrangement of one locus in the hybrids. Table 5.1 shows that the utilization of the *laevis* and *gilli* segments did not differ significantly in the six populations tested by sequencing. If there is any preference, it would be in the LG15 line where the *laevis* locus is utilized in a higher proportion of sequences. Although this is a limited sample, there does not seem to be a severe bias in locus usage but we must be mindful about interpreting any measure for biases Vβ segments which are only expressed from the *laevis* locus (see chapter 2 section 2.3.3). This supports the usage of the hybrids for repertoire comparison, because repertoire differences are unlikely to be due to peculiarities of the hybrids.

5.4.3.3 Dβ usage

Significant differences in the usage of the two *Xenopus* Dβ segments have not been observed. However, Dβ1 is used much more frequently than Dβ2 in all populations. Dβ2 is associated with Jβs 2, 3, 4, 5, 6, 7, 18, 19, 20 and 22 but is found predominantly rearranged to Jβ3. Of a sample of 44 Dβ2 containing sequences, taken from repertoire and ontogeny data, fully half are rearranged to Jβ3. All Vβ segments except Vβs 13 and 16 are associated with Dβ2.

5.4.3.4 Jβ usage

22 Jβ segments have been defined in LG hybrids. The *gilli* and *laevis* loci contain 13 and 19 Jβ segments respectively (see chapter 2). Jβ expression was studied in the same small subset of sequences cited in table 5.1.

Jβ segments are not employed equally. Jβ1 was not found in over 450 sequences. In fact, in all *Xenopus* TCRβ clones sequenced during both this study, the ontogeny study detailed in chapter 4, and the original project in which the TCR β chain was cloned ([77] and I. Chretien unpublished results), Jβ1 was only found twice. Jβ usage is remarkably similar between LG3 and LG15 lines (figure 5.4). Jβ3 and 8 are found most often in thymus derived sequences, whereas Jβ2, 9, 10, 11, 12 and 13 are used at a low, relatively stable level by all populations.

Significant differences are found in the thymic usage of Jβ segments in the LG15 line. Jβ5 is significantly scarcer in adult thymus than tadpole thymus (figure 5.4A). A comparison of the Jβ usage in the splenic repertoires does not show any significant differences (figure 5.4B). In the LG3 line, Jβ7 usage is lower in adults than in tadpoles. Jβs 15 and 17 are only found in sequences from LG3 adult thymus.

In LG15 tadpoles there is no significant shift in the usage of Jβ segments between the thymus and spleen (figure 5.4C). In the adult situation, the absence of Jβ4 in the periphery was the only statistically significant difference.

5.4.3.5 Vβ usage

It is immediately apparent that all Vβ families are not equally well represented in the repertoire (figure 5.5). In making comparisons, it is important to realize that membership in a Vβ family is defined by a 75% homology, thus making cross-hybridization between members of a family inevitable. We however did not see cross-hybridization between different families of segments. Therefore, Vβ1, 6 and 8 with 3, 2 and 3 members respectively, may seem more prevalent when compared to the rest of the repertoire. However, from figure 5.5 we can see that this was not always the case. While BV1 and BV6 are more highly represented in the thymic repertoire (fig. 5.5A), BV8 is not more prevalent in the repertoire than many of the single member families.

The hybridization procedure used for screening, could conceivably bias the observed repertoire through introducing experimental error. Such error must be minimal for the proportional usage of Vβs 2,3,8,11, 12, 14, 17, and 18 is remarkably similar in all of the repertoires studied, despite the fact that results were obtained from different cDNA libraries and different cohorts of animals. However, there were also some notable and statistically significant differences observed: developmental (tadpole-adult), selection (thymus-periphery) and strain specific (tadpole-tadpole and adult-adult) differences.

5.4.3.5.1 The thymic "unselected repertoires" differ between tadpoles and adults

Because both animal lines possess the same TCR loci, the rearrangement dynamics, and thus the preselected repertoire should remain relatively constant. This is true for the majority of Vβs. Data in figure 5.5A show that the proportional usage of Vβ 2, 3, 5, 8,10,11,12,13, 14, 16, and 17 are not statistically different in the thymic repertoires of tadpoles and adults in both animal lines. There are, however significant differences in the thymic repertoires of tadpoles and adults. In the LG15 line there are proportional differences in Vβs 1, 4, 6, 9, and 18 between adults and tadpoles. In LG3, this developmental difference is only significant in the case of Vβ15. When we compare the tadpole thymic repertoires in the two frog lines, the only significant difference is in Vβ1 usage, and a comparison of the adult repertoires shows no notable differences. Thus, we see developmental differences in the observed thymic repertoires of tadpoles and adults, rather than differences attributed to the two MHC backgrounds.

5.4.3.5.2 Thymic repertoire differences may be partially attributed to the mixture of selected T cells present in the thymus

The thymus preparations are not entirely pre-selected cells but a mixture of cells undergoing selection and selected cells that have not yet migrated to the periphery.

In LG3 tadpoles and adults, the usage of Vβ15 in their respective peripheral repertoires does not vary significantly (figure 5.5C). In LG15 tadpoles the thymic and peripheral Vβ1 and 4 usage does differ significantly whereas usage of Vβ9 and 18 does not (figure 5.5D). For LG15 adults Vβ1, 4, 9, and 18 do not show notable differences. Thus, it is possible that the mixture of cells present in the thymus leaves us measuring a partially selected repertoire, but the differences in usage of Vβ1 and 4 in LG15 tadpoles suggests that this does not entirely mask differences in the thymic and peripheral repertoires.

5.4.3.5.3 Are there significant differences in the thymic and splenic repertoires?

Next we asked if the imprint of selection on the "raw" unselected repertoire is apparent by comparing the thymic and peripheral repertoires in each population. The only notable change in the thymic and peripheral repertoires of LG3 tadpoles is in the proportional usage of Vβ1 (figure 5.5C). In LG15 tadpoles Vβ1 and 4 also show significant changes (figure 5.5E). In LG3 adults Vβ1 and 5 showed notable shifts in proportional usage while in LG15 there were no such shifts detected (figure 5.5F). Thus, the differences between the thymic and peripheral repertoires seem minimal. This may also be attributed to the mixture of populations found in the thymus.

5.4.3.5.4 The splenic repertoires

LG3 and LG15 are two sibs that share the same TCR locus (see chapter 2) but which differ in their MHC alleles and at many other loci. This provides a very different set of self-peptides to be presented to T cells in the two frog lines. The effects of these differing selection constraints on the T cell repertoire can be evaluated in three ways. First, one can measure the effect of the transition to adulthood on the Vβ usage within each line. This should be influenced by the appearance of MHC class I at metamorphosis (i.e., a tadpole-adult comparison). Second, the differing genetic backgrounds on the adult Vβ repertoire can be determined (i.e., adult-adult comparison). Third, the influence of selection in the absence of class I MHC on the Vβ repertoire can be determined by comparing splenic tadpole repertoires between the LG3 and LG15 lines (i.e., tadpole-tadpole comparison).

5.4.3.5.5 Tadpole vs. adult

In LG3 two significant differences in Vβ usage were found during ontogeny: the usage of Vβ5 and Vβ15 was 13 and 2.5 fold higher in adult spleen than in tadpole spleen. Other variations such as a 3 fold lower usage of Vβ8 in adults and a 5-fold increase in Vβ16 usage relative to the tadpole, were not statistically significant.

The situation in LG15 is quite different as there were no statistically significant differences in Vβ usage between the larval and adult repertoires.

Therefore the changes in peripheral repertoire during ontogeny are not the same in the two lines.

5.4.3.5.6 Adult vs. adult

There were differences between the adult spleen repertoires of LG3 and LG15. Again, Vβ5 and Vβ15 are involved. Vβ5 usage in LG3 is 2.6 times higher than in LG15. Vβ15 usage in LG3 is also 2.1 times higher than in LG15. These differences are very significant, both with p values below 0.001, and may involve both class I and class II MHC differences between the two lines.

5.4.3.5.7 Tadpole vs tadpole

In addition to the line specific repertoire differences during ontogeny, the LG3 and LG15 splenic repertoires can differ significantly between tadpoles. Vβ3 is used 2.5 times more in the LG3 repertoire than in the LG15 repertoire. This may be especially significant. The comparison of Cβ usage in table 1 suggests that there may be a slight bias towards usage of the *laevis* constant region (locus) in LG15. Vβ3 is only expressed from the *laevis* locus, because the *gilli* Vβ3 is most likely a pseudogene (see chapter 2). Here we find Vβ3 used more often in the LG3 line suggesting that there is no bias in Cβ usage or that there is a strong bias in the repertoire for Vβ3. In fact both the LG3 tadpole and adult peripheral repertoires use Vβ3 more often than the LG15 splenic repertoires, suggesting that this is a difference dependant on MHC alleles present in LG3 or the peptide landscape differences between the two lines. This pattern is not seen in the thymic repertoires of LG3 and LG15.

Vβ9 usage is 6-fold higher in the LG15 line than in the LG3 line. The differences in Vβ1 usage in the LG3 and LG15 splenic tadpole repertoires are almost significant (p>0.10). A bigger sample of the repertoire needs to be screened to address if this is a significant difference.

These differences may be due to selection on different class II alleles in the absence of classical and non-classical MHC class I molecules during tadpole life.

5.4.3.5.9 LG3 vs. LG15 splenic repertoires

LG3 and LG15 share a TCR locus but the MHC and many of the self-peptides presented on MHC will differ between the lines. There are several differences in repertoire which are consistent in both the tadpoles and adult peripheral repertoires of a line. For instance Vβ1 usage seems lower in LG3 peripheral repertoires than in LG15 (figure 5.5B). BV3 is used more often in splenic LG3 repertoires than in LG15 spenic repertoires (figure 5.5B). These comparisons are not all statistically significant but such shared differences between adults and tadpoles likely reflect Vβs which are more dependant on particular class II MHC alleles for selection or deletion. It is clear that the same TCR locus will produce distinct TCRβ repertoires when selected on particular MHC backgrounds.

5.5 Discussion

This study gives us our first glimpse of the proportional usage of TCR segments in a poikilothermic vertebrate. As well it is the first comparison of a larval (see section 4.3.1) and adult T cell repertoire in a poikilothermic vertebrate. This work parallels studies done in mice and humans outlining the nature of a normal TCR repertoire as well as studies of the repertoire of MHC class I knockout mice. This study was carried out to determine if there are significant differences in the proportional usage of Vβ segments in the repertoires, and to determine if these differences are more correlated with MHC background, developmental stage or thymic vs. peripheral repertoire.

5.5.1 Anchored PCR allows small repertoires to be measured

The use of the anchored PCR approach allows the repertoires of even very small T cell populations to be reliably measured. Adding a known 5' adapter sequence via template switching of an RNase H$^-$ reverse transcriptase (see figure 5.1) is much more efficient than traditional poly-G tailing with TdT or ligation based methods. All attempts to use ligation based methods resulted in libraries with significant bias. Vβ specific primers always amplified more rearrangements from these libraries than RACE amplifications showing that the addition of adapters through ligation is far too inefficient to measure small repertoires. Therefore, anchored PCR using the Smart RACE system is the method of choice for future repertoire studies.

As with all sensitive methods, one must be very cautious to avoid contamination of samples. Extensive controls were done for each reaction to ensure that neither the reagents or the library had been contaminated by plasmids present in the lab. Vβ 19 was not included in this study. Preliminary sequencing data suggest that Vβ 19 is quite rarely used and therefore its absence is unlikely to bias the repertoire proportions.

In repertoire studies there is always a risk that an individual undergoing an infection could have a very skewed repertoire due to the proliferation of a subset of T cells (oligoclonal expansion). We attempted to minimize this risk by constructing RACE libraries from RNA pooled from 5-10 genetically identical individuals that were sacrificed at different times and were from different breedings. Thus, if such an effect exists, it is averaged with the repertoires of several other individuals. As well, populations from which clones were randomly sequenced showed no obvious oligoclonal expansions (i.e. repetitions of the same sequence). This ensures that individual differences will not bias the repertoire, but also ensures that subtle differences in the repertoire will be overlooked.

The comparison of thymic repertoire with the splenic repertoire may also be muddied by the presence of both unselected and selected T cells in the thymus. We currently do not have the reagents to sort these populations using FACs. The proportion of selected to unselected T cells is likely to change as the thymus becomes bigger during development which may account for some of the differences in thymic pre-selected repertoire.

The diversity of each library was tested by internal amplification of the RACE band with Vβ specific primers as is shown in figure 5.2 and by sequencing. These methods can provide a sampling of the variability in the library but cannot determine if the repertoire is more subtly biased. In future studies, immunoscope should be carried out to determine if the repertoire of each library has a normal distribution of CDR3 lengths in the population implying that the repertoire is not skewed by expansions caused by immune responses. This would ensure that the hybridization results would be representative of that population.

However, immunoscope analysis is also biased by limiting samples of T cells for it is reliant on Vβ specific amplifications. Future immunoscope analysis of small populations may benefit from the modified Smart RACE protocol presented here. When working with small numbers of T cells in the ontogeny study presented in chapter 4, it became clear that there was a problem with dilution of limiting samples. For instance, if one needs to characterize the diversity of a population of 50-200 cells, and to carry out 19 Vβ specific PCR reactions in order to determine the diversity in the sample, significant diversity may be lost to the assay by dividing the template. This happens when looking at T and B cell ontogeny in *Xenopus*, but it is conceivably also a problem when characterizing other small T cell populations such as IELs or specific subsets of FACs sorted cells. I suggest that this efficient RACE protocol could be used to amplify the entire repertoire without introducing bias, so that sufficient template is available for immunoscope spectrotyping.

Despite the potential limitations of using this experimental approach, we do see a remarkably uniform usage of Jβ and Vβ segments in all studied populations.

The sample size for the collected Jβ repertoires is not large enough to draw solid conclusions. We see that although Jβ 18 is not found in the LG15 adult thymus it is nevertheless detected in the periphery so the sample size is still too small to determine the specifics of the Jβ repertoire. Because there is only a single Cβ segment in the *Xenopus* TCR β locus, we had not expected to find so many Jβ segments and had not anticipated sequencing enough samples to make a study of Jβ usage statistically significant. What we can take from this data is a general finding of non-random usage of Jβ segments as has been found in mice [82] and humans [91].

All Vβ segments, with the exception of Vβ 2 in LG3 adult thymus, and Vβ 10 in LG3 adult spleen and LG15 tadpole spleen, can be used in the thymic and peripheral repertoires of adults and tadpole of both frog lines. These three exceptions are most likely due to our sample not being large enough, for they are from Vβ families that are generally not highly expressed. The finding that the proportional usage of Vβ 2, 3, 5, 8, 10, 11, 12, 13, 14, 16, and 17 are not statistically different in the thymic repertoires of tadpoles and adults in both animal lines further validates the use of this experimental approach. One concern that we had was that multimember Vβ families such as Vβ 1, 6 and 8 would be over-represented in the repertoire. However, Vβ 8 is not highly used therefore the number of segments does not always translate into higher representation in the repertoire. This is also the case in mice and humans (reviewed in [2]). The *Xenopus* normal TCR Vβ repertoire is therefore much like that of mice and humans where all Vβ families are expressed during both fetal and adult life [20, 31, 34, 66, 83].

The repertoire is also not significantly different from that measured in class I MHC knockout mice. CD8$^+$ cells selected on non-classical MHC class I molecules from these mice were sorted by fluorescence activated cell sorting FACs. The TCR α and β repertoires of these cells were extremely variable. In H-2 K^{b}D$^{b\ -/-}$ double knockout as well as the H2K$^{b\ -/-}$ and H2D$^{b\ -/-}$ single knockout mice all Vβ segments were used and the immunoscope profiles showed a Gaussian curve characteristic of a normal repertoire of CDR3 lengths [52].

Like classical class I knockout mice, *Xenopus* tadpoles can express all Vβ segments. However, the only repertoire difference detected in comparing "normal"

(C57BL/6 mice lacking H-2L^b) mice with the MHC Ia knockout mice was the increased usage of Vβ 5.1 in the knockout mice [52]. In *Xenopus*, significant differences in Vβ5 and 15 were detected in LG3 tadpoles (MHC$^-$ or lo) and adults (MHChi) although no corresponding difference was observed in LG15. This could be interpreted in several ways. First, the significant difference is very significant because an effect is seen in the mixture of CD4 and CD8 cells rather than the sorted CD8 samples studied in the mouse repertoire study. Second, the result shown in figure 4.10 indicates that MHC class Ia transcripts are present at even very early time-points in tadpole development were carried out on LG15 animals. There could be a difference in the expression of class I in the two LG lines. Perhaps LG3 has a lower amount of class I expression leading to the differences between tadpoles and adults where in LG15 the difference is not as profound. Further work will need to be done to clarify this matter.

The absence of surface class I expression until late in tadpole life has been an intriguing mystery. Here we asked if perhaps the lack of class I produced a stable selection for a TCR Vβ or Jβ segment in the rearrangements. Such a "canonical" receptor should be found in both the LG3 and 15 animal lines which differ in their MHC loci, but not at the TCR. Only in the case of Vβ1 was such a difference seen. There are other factors that could bring about this skewing, for instance a bias in Vβ usage in CD4$^+$ and CD8$^+$ cells. Perhaps the bias results from the lower number of CD8$^+$ T cells in tadpoles. In any case, the developmental differences in Vβ usage are not generally the same in both frog lines, which suggests that the differences in the selecting MHC profoundly affects the repertoire.

5.5.2 The repertoires of human identical twins

The human TCR β repertoire is much more similar in twins than in unrelated individuals [83]. Furthermore, the repertoire of twins differed from MHC identical siblings [84]. This was interpreted as evidence of a strong genetic component in the initial formation of the repertoire. The repertoire seemed more dependent on the TCR locus present than the selecting MHC [84]. Differences in the self-peptides presented in the individuals must also play some role as well. This is an interesting observation, although, as in most studies done in humans, not enough individuals were studied to draw any firm conclusions. This hypothesis could be tested in *Xenopus* by using other LG siblings that are matched at the MHC but differ at the TCR locus.

5.5.3 Developmental differences

It is interesting to note that the screening technique shows us that the thymic repertoires of the two strains are remarkably similar in age matched populations. We expect that the thymic repertoires of animals with the same TCR β locus should not differ. It is perplexing that the thymic repertoires of tadpoles and adults differ in both lines. This may imply a subtle difference in recombination between tadpoles and adults. This has not been observed in mice or humans [2], and should be investigated further in *Xenopus*. This may be due to the free-living lifestyle of the tadpole. A comparison of the early repertoires of other species, such as fish and other amphibians that are free-living at early points in their development may prove fruitful. Especially interesting will be a comparison with the repertoires of marsupials which also are "born" at points early in their development.

5.5.4 Genetic differences

This was also seen when comparing the peripheral repertoire of adults and of tadpoles. The splenic peripheral repertoires of tadpole and adult differed in the LG3 lines but not in the LG15 line suggesting that MHC or other genetic difference between the lines biases the repertoire. It is important to note that the usage of the majority of Vβs are quite similar in all populations tested, showing that there is a variable baseline repertoire which does not change much with genetic selection differences. It would be intriguing to test if this is due to the shared TCR locus.

5.6 General conclusions

We can conclude from this study that LG3 and LG15 lines are good models in which to study the effect of MHC expression and development changes on the T cell repertoire. Large numbers of genetically identical animals can be produced from these lines allowing us to perform experiments on a genetically uniform background. In both LG3 and LG15, the *laevis* and the *gilli* TCR β loci undergo rearrangement and the their repertoires are very similar both for Jβ and Vβ usage for the majority of segments. As is the situation in mice and humans, Jβ and Vβ segments are non-randomly used in the repertoire. This appears to be the case in the thymus and most likely is a characteristic of the rearrangement process.

Significant differences in the repertoires are seen. There are greater differences between adult and tadpole thymic repertoires than there are when only tadpole or adult repertoires are compared between LG3 and LG15. This suggests that there may be slight but intrinsic developmental differences in the repertoire. This could be due to the lack of MHC class I in tadpole life or it could be due to the lack of TdT and therefore the shorter CDR3 junctions in tadpoles. This study did not show major perturbations of the proportional Vβ usage in the thymic and peripheral repertoires. This may be due to the use of the entire thymus that includes both selected and immature unselected cells. The differences between the truly unselected and peripheral repertoires in such a case would be muddied, but large differences would still be detected. Therefore, this study may underestimate the true imprint of selection on the unselected T cell population. It seems clear that there are no canonical patterns of Vβ or Jβ usage present, whatever the MHC background. The peripheral repertoires showed significant differences between lines in tadpoles and in adults, suggesting that MHC background and the "landscape" of peptides presented have an important effect on the repertoire.

As reagents become available which will allow us to sort different T cell populations in the thymus this issue will need to be re-addressed. These results show that tadpoles possess as diverse a repertoire as adults confirming results presented in chapter 4. In interpreting this diversity it is important to remember that each library represents between 10 and 20 individuals, so individual variability may still be more limited in tadpoles. PCR on individuals or even pools of 5 individual 24 dpf tadpoles did not always amplify rearrangements employing all 19 Vβ segments. I am inclined to conclude that this is due to the limited numbers of cells in these animals, because of the non-random usage of segments, the rarer Vβ segments are less likely to be represented. However, PCR is a fallible technique and negative results are difficult to interpret. The sheer repetition of these studies makes this less likely but cannot rule it out entirely.

Only general conclusions can reasonably be taken from this work. This repertoire study is low resolution, providing a few generalizations and a baseline for future repertoire studies. We know that there are significant differences in the TCRβ repertoire, some of which seem dependent on developmental differences in MHC expression and others more affected by MHC differences between animal lines. Based on these results we can further narrow the population of T cells tested or change the selection environment for the T cells by introducing class I MHC expression in tadpole life or increasing the dosage of class I expression by studying lab generated polyploid *Xenopus* lines.

Sample	C_L	C_G	total
LG3 tadpole thymus	27	27	54
LG3 adult thymus	34	35	69
LG15 tadpole thymus	46	16	62
LG15 tadpole spleen	33	19	52
LG15 adult thymus	38	14	52
LG15 adult spleen	26	20	48

Table 5.1 Usage of Cβ domains in *Xenopus* LG hybrids

Clones were randomly sequenced from the RACE libraries shown. Sequences were divided into those with a *laevis* "allele" (C_L) or a *gilli* allele (C_G) of the Cβ domain. The total number of sequences is shown at right.

Table 5.2 Summary of statistically significant differences in TCR β repertoire

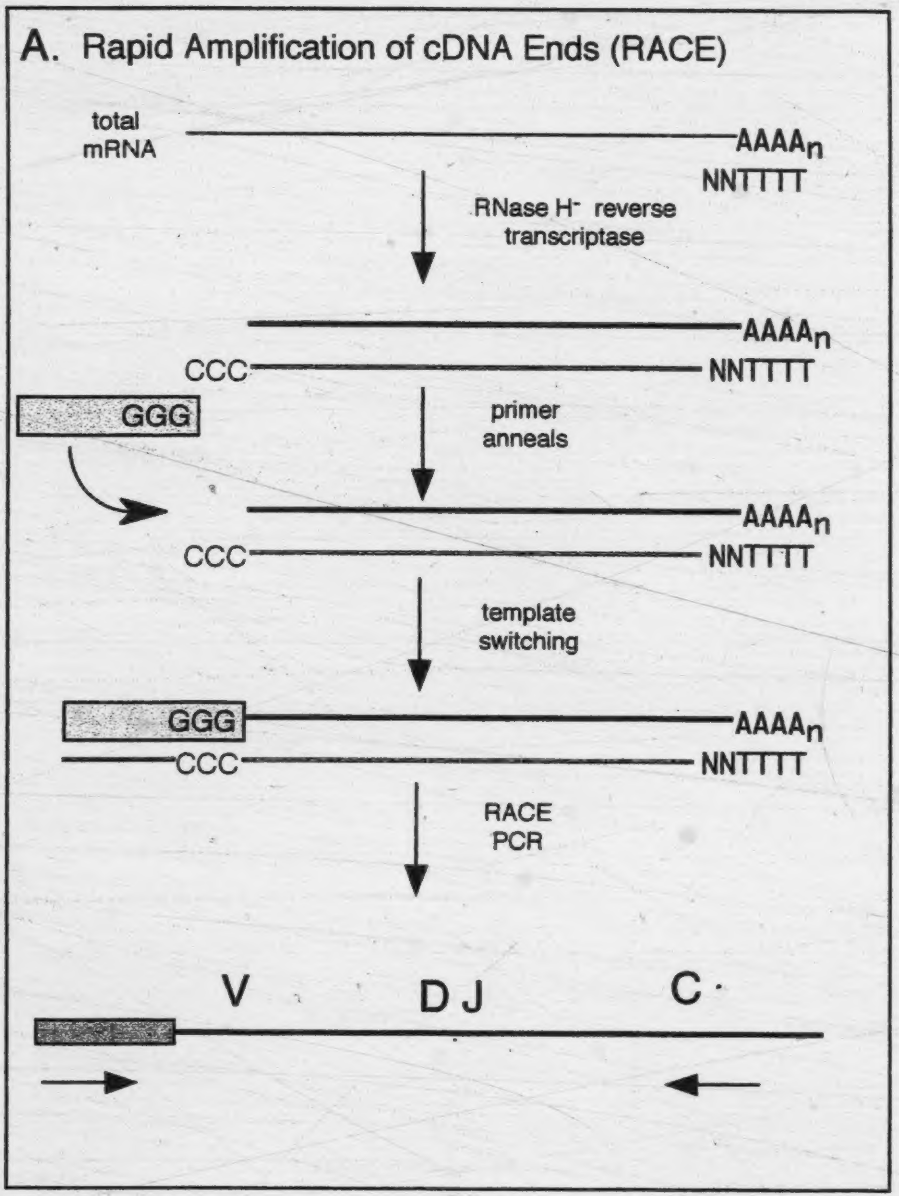
The findings presented in figure 5.5 are summarized here. Development refers to differences between the tadpole and adult repertoires. Selection refers to repertoire differences between the thymic "unselected" repertoire and the splenic selected repertoire. Genetics refers to differences in repertoire seen between LG3 and LG15 lines that share the same TCR locus but differ at the MHC loci and in presented self-peptides. Numbers refer to Vβ segments. Zero (0) means that there were no significant differences observed in that comparison.

Table 5.2 Repertoire Summary

Comparison		development
Thymus-Thymus		
tadpole-adult	LG3	15
tadpole-adult	LG15	1, 4, 6, 9, 18
Spleen-Spleen		
tadpole-adult	LG3	5, 15
tadpole-adult	LG15	0
		selection
Thymus-Spleen		
tadpole	LG3	1
tadpole	LG15	1, 4
adult	LG3	1,5
adult	LG15	0
		genetics
Thymus-Thymus		
tadpole - tadpole	LG3-LG15	1
adult - adult	LG3-LG15	0
Spleen-Spleen		
tadpole - tadpole	LG3-LG15	3, 9
adult - adult	LG3-LG15	15

Figure 5.1 Construction of SMART RACE libraries

Rapid amplification of cDNA ends (RACE) involves the addition of a known adaptor sequence to the 5' end of unknown cDNA sequences allowing the 5' end of genes to be quickly cloned without need for hybridization or sequence information. The procedure uses an anchored poly-T primer to prime the production of a first cDNA strand by an RNase H- reverse transcriptase. Under specific buffer conditions, the transcriptase will add 3 to 4 extra untemplated C residues at the end of the template strand. An adaptor with 4 to 5 G residues at its 3' end then anneals to the C residues. The reverse transcriptase then switches templates and copies to the end of the adaptor thus adding a known sequence to the 5' end of most cDNAs in a library. Here, the RACE technique allows the amplification of all TCR β rearrangements in a library without the bias of V specific primers. SMART RACE is available as a kit from CLONTECH and the above is redrawn from the manual for this kit.



A. Rapid Amplification of cDNA Ends (RACE)

total mRNA
AAAA$_n$
NNTTTT
RNase H$^-$ reverse transcriptase
AAAA$_n$
CCC
NNTTTT
GGG
primer anneals
AAAA$_n$
CCC
NNTTTT
template switching
GGG
AAAA$_n$
CCC
NNTTTT
RACE PCR
V
D J
C

Figure 5.2 An example of a typical RACE reaction

PCR is used to amplify all TCR β rearrangements in A. day 24 spleen (24S), day 24 tadpole thymus (24T), and in B. adult spleen (AS) and adult thymus (AT) RACE libraries. Shown is a typical resulting band after two rounds of amplification using nested primers (see materials and methods). Controls include: water as template (W) and plasmid controls for both spleen (PLS) and thymus (PLT) libraries. Although the spleen "bands" are very difficult to see, they do contain enough material to subclone. Over amplification resulted in overrepresentation of a single sequence or a small number of sequences (data not shown). Tadpole RACE bands include a diverse mix of rearrangements. C. Aliquots of tadpole RACE bands shown in A were diluted and used as template for Vβ specific amplifications. Vβs 11 through 19 are tested and water controls are shown. For the spleen sample, the Vβ12 reaction failed due to a technical problem (the reaction tube was not properly closed during PCR). Subsequent hybridization of colonies derived from this library did not detect the presence Vβ12. D. Adult RACE bands include a diverse mix of rearrangements. Vβs 1 through 11 are tested and plasmid (PL) and water controls are shown. The reaction with Vβ4 did not amplify in this experiment although subsequent sequences and hybridizations did show the presence Vβ4.

Figure 5.2

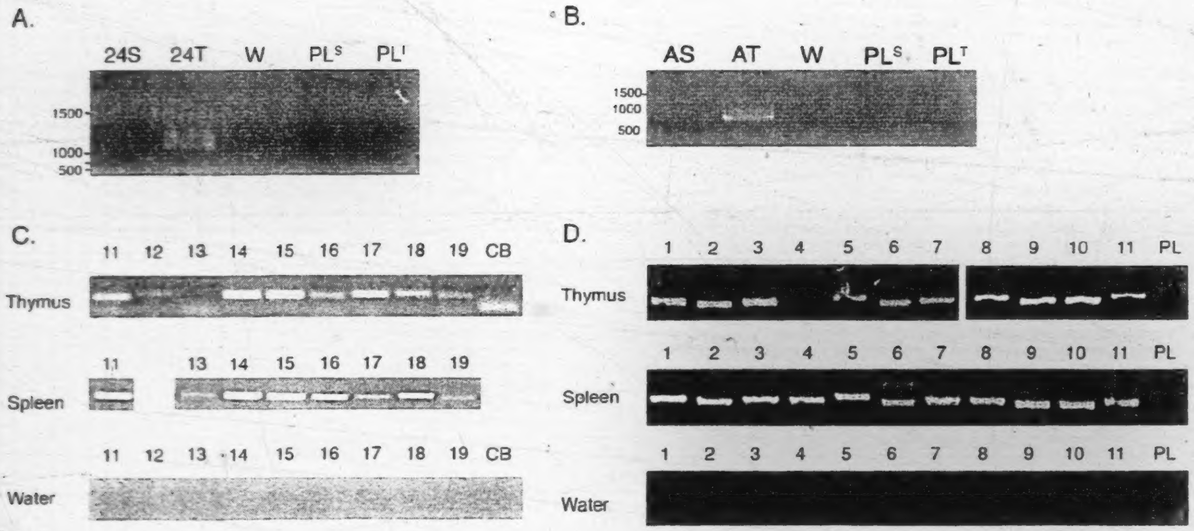

A.
24S
24T
W
PL^S
PL^T
1500
1000
500
B.
AS
AT
W
PL^S
PL^T
1500
1000
500
C.
11 12 13 14 15 16 17 18 19 CB
Thymus
11 13 14 15 16 17 18 19
Spleen
11 12 13 14 15 16 17 18 19 CB
Water
D.
1 2 3 4 5 6 7 8 9 10 11 PL
Thymus
1 2 3 4 5 6 7 8 9 10 11 PL
Spleen
1 2 3 4 5 6 7 8 9 10 11 PL
Water

Figure 5.3 Experimental Design

RACE libraries were constructed from total RNA derived from the thymus and spleen of tadpoles and adults of both the *Xenopus* LG3 and the LG15 animal lines. These libraries were constructed in duplicate using genetically identical animals from different breedings. The libraries were used as template for amplifications of all TCR β chains in the library. These PCR products were then subcloned and the diversity of each library confirmed by sequencing the inserts from a small subset of randomly selected clones. Multiple lifts were taken from 5 plates per library and these filters were hybridized sequentially with probes for each of 18 *Xenopus* Vβ families. The duplicate library for LG15 tadpole thymus was not screened because of time constraints.

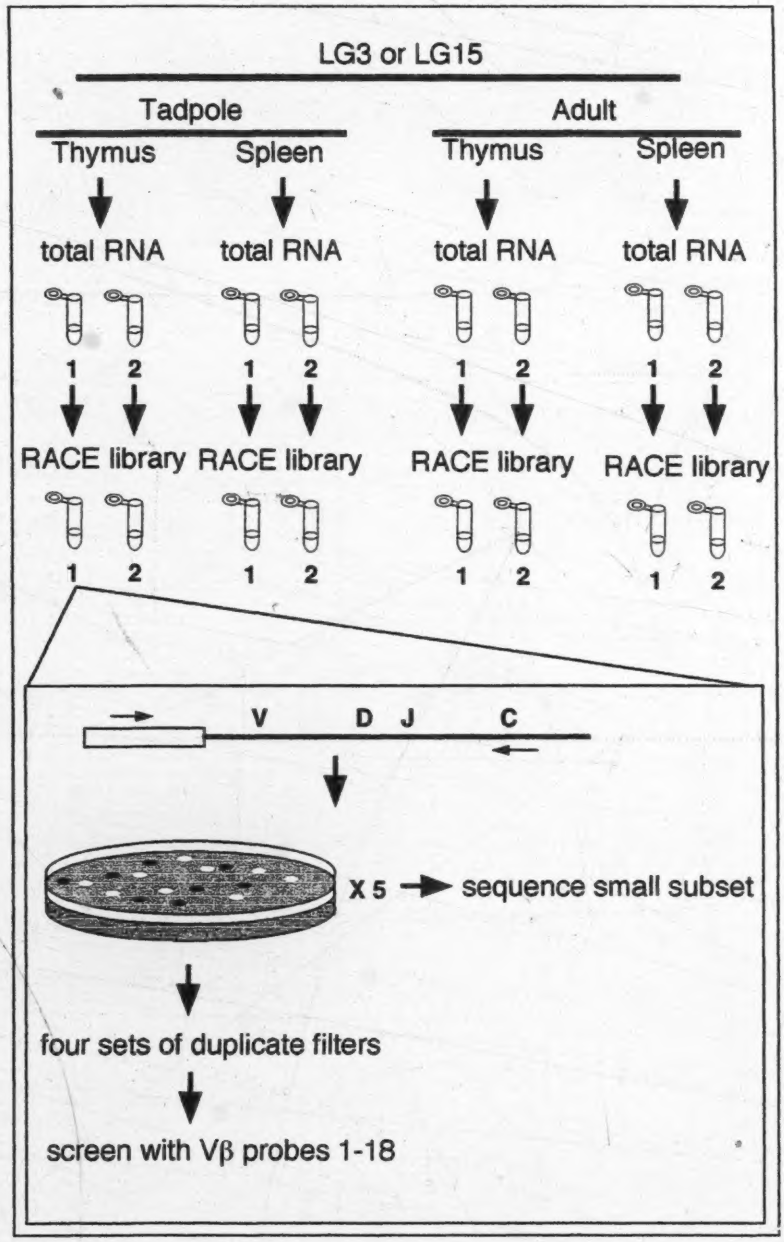

LG3 or LG15
Tadpole
Adult
Thymus
Spleen
Thymus
Spleen
total RNA
total RNA
total RNA
total RNA
1
2
1
2
1
2
1
2
RACE library
RACE library
RACE library
RACE library
1
2
1
2
1
2
1
2
V
D
J
C
X 5
sequence small subset
four sets of duplicate filters
screen with Vβ probes 1-18

Figure 5.4 Proportional Jβ usage

Proportional usage of the 22 *Xenopus* Jβ segments was calculated from TCR β sequence samples obtained from the following RACE libraries: (i) LG3 tadpole thymus; (ii) LG3 adult thymus; (iii) LG15 tadpole thymus; (iv) LG15 tadpole spleen; (v) LG15 adult thymus; and (vi) LG15 adult spleen. In A, the thymic Jβ repertoires are compared. In B the splenic Jβ repertoires are compared. In C and D, the adult thymic and splenic Jβ repertoires are compared. Significance at the 95% confidence level ($p < 0.025$) as tested by the Fisher exact test, is denoted by an asterisk (*).

Figure 5.4

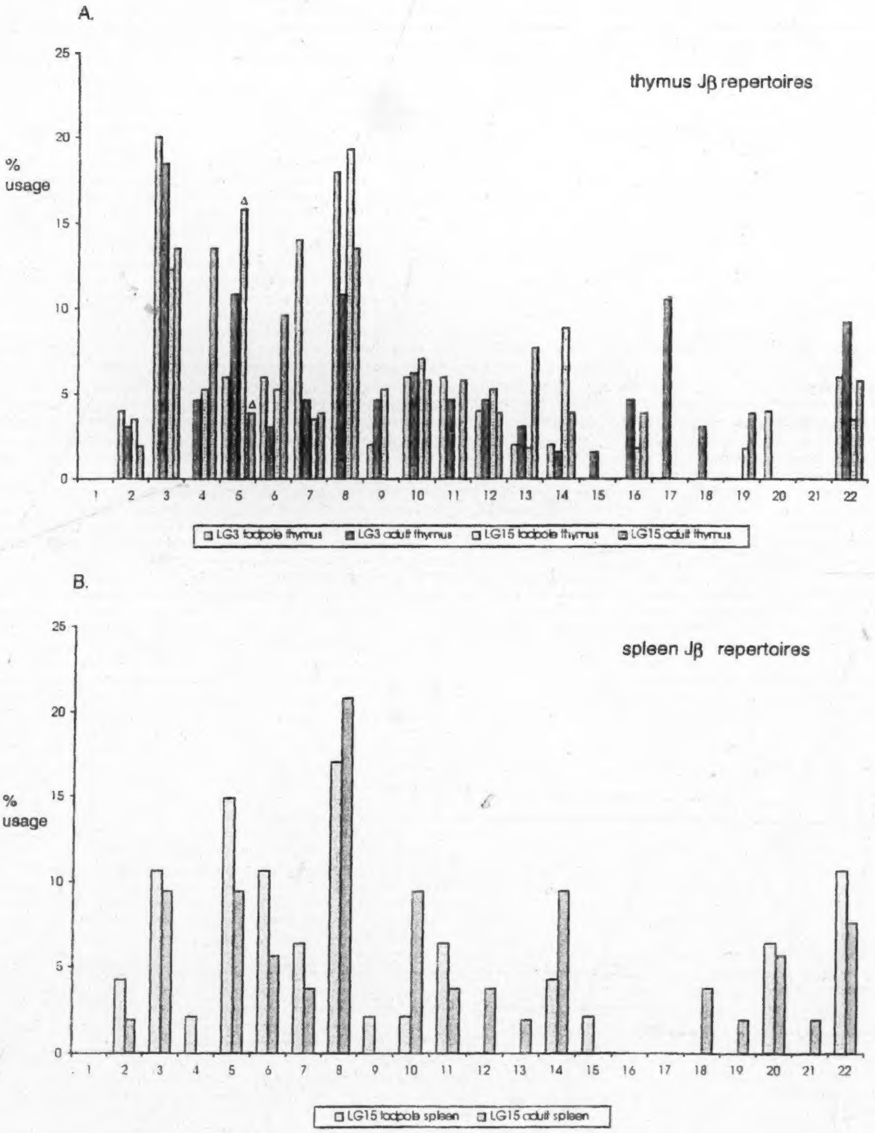

A.
thymus Jβ repertoires
% usage
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
Δ
Δ
□ LG3 tadpole thymus
▨ LG3 adult thymus
□ LG15 tadpole thymus
▨ LG15 adult thymus
B.
spleen Jβ repertoires
% usage
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
□ LG15 tadpole spleen
▨ LG15 adult spleen

Figure 5.4

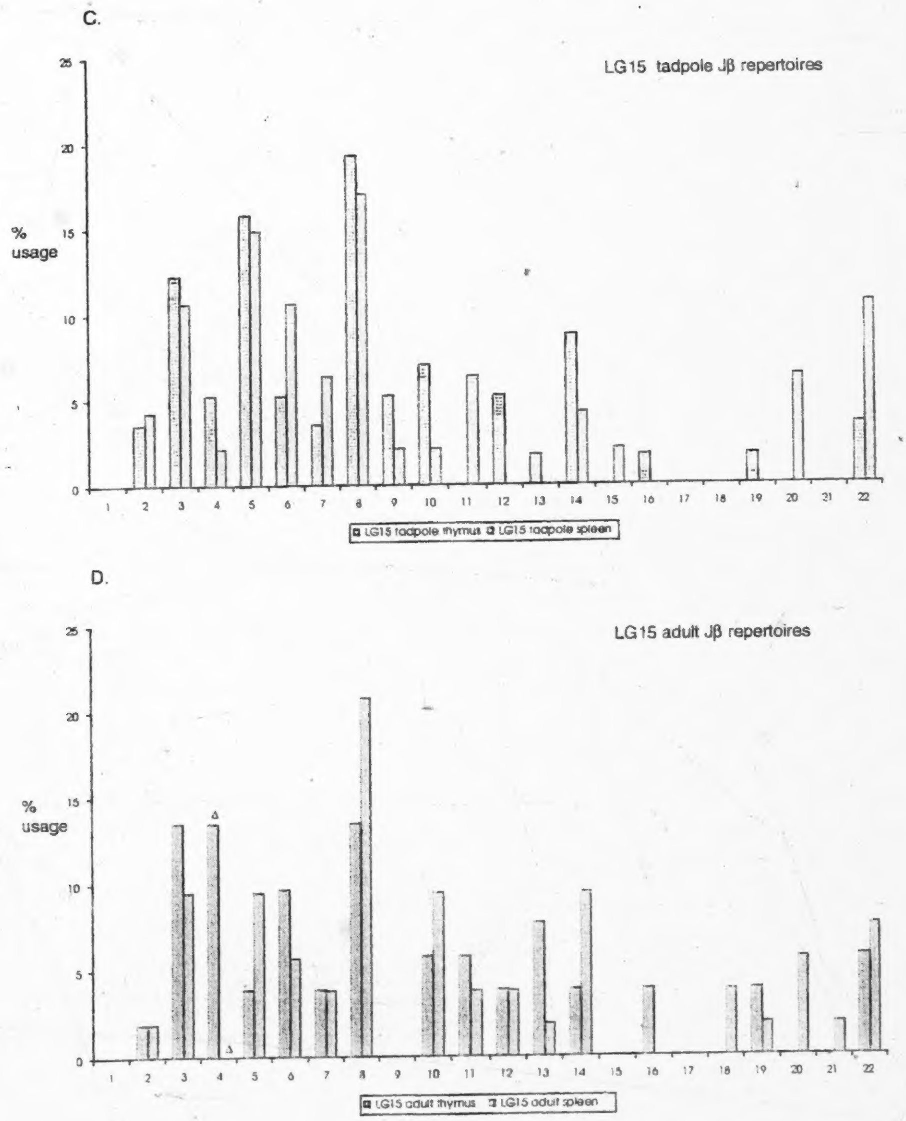

C.
LG15 tadpole Jβ repertoires
%
usage
0
5
10
15
20
25
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
LG15 tadpole thymus
LG15 tadpole spleen
D.
LG15 adult Jβ repertoires
%
usage
0
5
10
15
20
25
Δ
Δ
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
LG15 adult thymus
LG15 adult spleen

Figure 5.5 Proportional Vβ usage

The proportional usage of 18 of 19 *Xenopus* Vβ segments was measured by subcloning a population of TCR β fragments amplified from a RACE library. Duplicate lifts of those colonies were hybridized with the probes shown on the x-axis. Proportional usage was calculated as the number of hybridizing colonies for Vβ1-18/ the sum of hybridizing colonies for all Vβs X 100. Data is presented in several groupings: A. thymic Vβ repertoires; B. spleen Vβ repertoires; C. LG3 Vβ repertoires; D. LG15 Vβ repertoires; E. tadpole Vβ repertoires; and F. adult Vβ repertoires. An X denotes missing data sets. Statistical significance is tested by a two-sided χ^2 test or, where counted values are below 5, with a Fisher Exact test. Significance of differences of the expression or each Vβ segment is denoted as follows: $p < 0.001$ Δ, ■ or *; $p < 0.005$ ● ; $p < 0.025$ for df=17. The total number of colonies measured for each library are as follows LG3 tadpole spleen, 523; LG3 tadpole thymus, 431; LG15 tadpole spleen, 648; LG15 tadpole thymus, 775; LG15 adult spleen, 659; LG15 adult thymus, 1372; LG3 adult thymus, 765; and LG3 adult spleen, 846. A complete table of values is presented in appendix

Figure 5.5

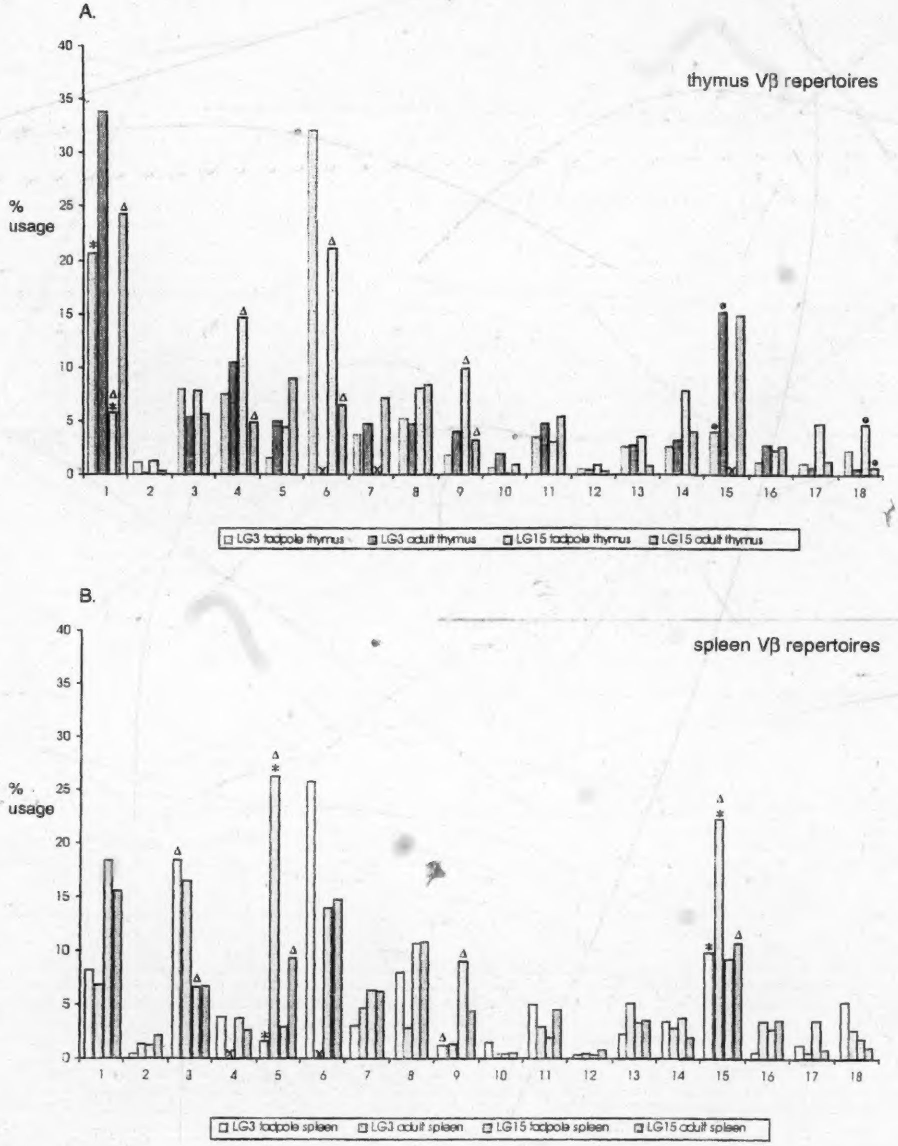

A.
40
35
30
25
20
15
10
5
0
% usage
thymus Vβ repertoires
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
LG3 tadpole thymus
LG3 adult thymus
LG15 tadpole thymus
LG15 adult thymus
B.
40
35
30
25
20
15
10
5
0
% usage
spleen Vβ repertoires
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
LG3 tadpole spleen
LG3 adult spleen
LG15 tadpole spleen
LG15 adult spleen

Figure 5.5

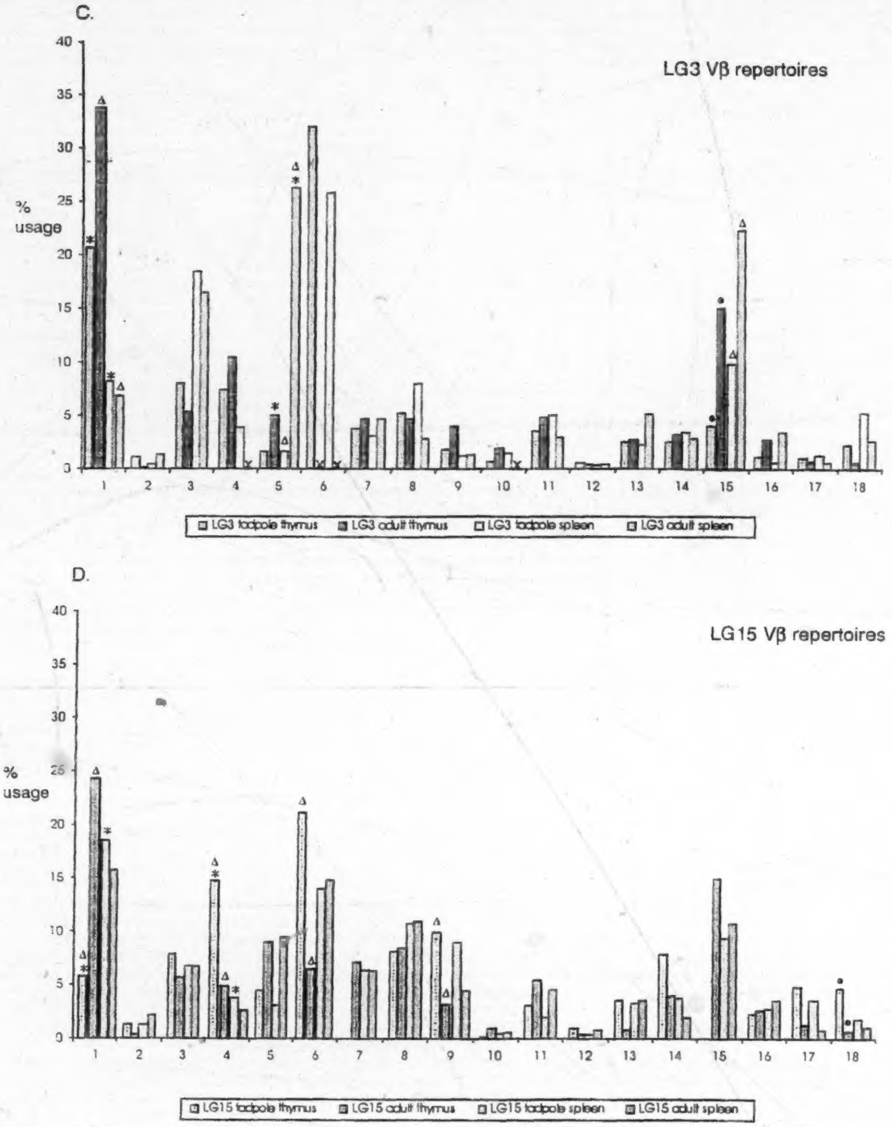
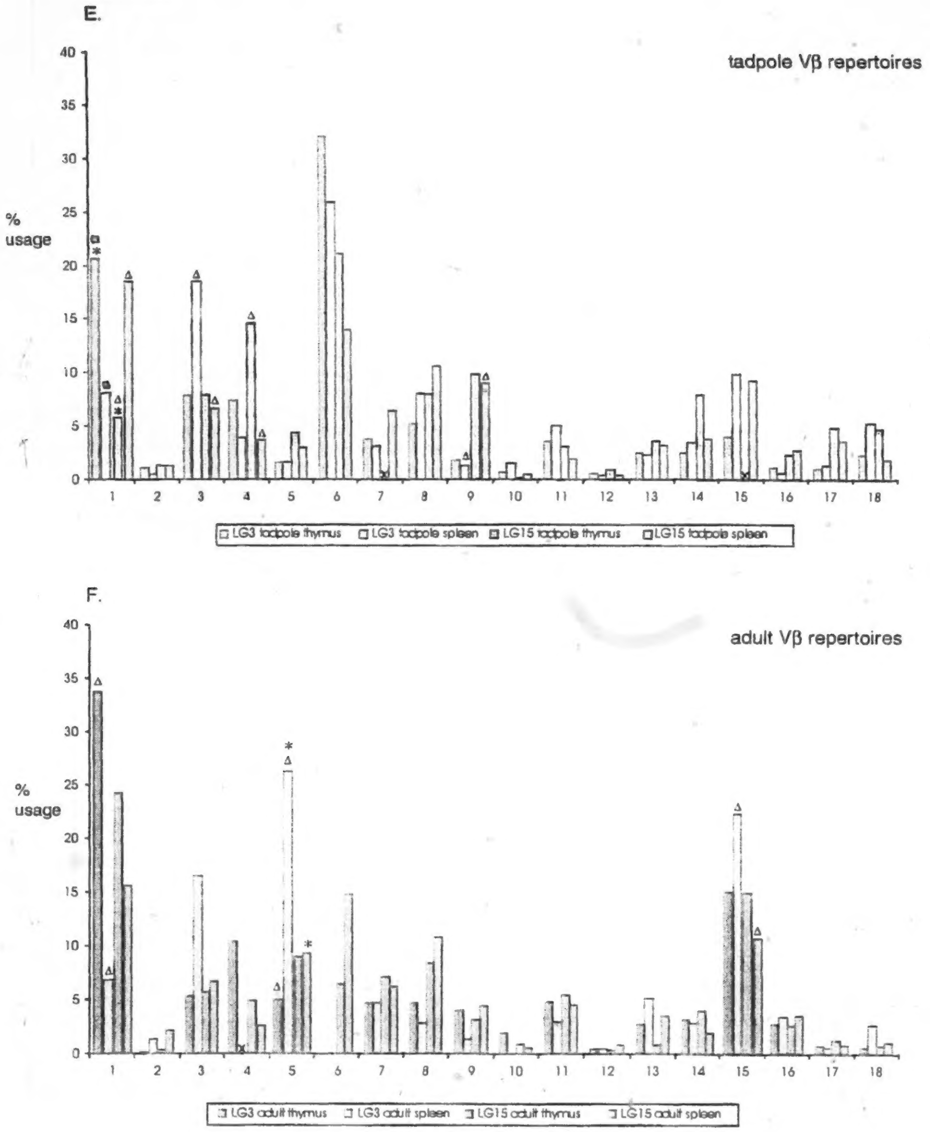

C.
LG3 Vβ repertoires
% usage
0
5
10
15
20
25
30
35
40
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
▨ LG3 tadpole thymus
▨ LG3 adult thymus
▨ LG3 tadpole spleen
▨ LG3 adult spleen
D.
LG15 Vβ repertoires
% usage
0
5
10
15
20
25
30
35
40
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
▨ LG15 tadpole thymus
▨ LG15 adult thymus
▨ LG15 tadpole spleen
▨ LG15 adult spleen

Figure 5.5


E.
tadpole Vβ repertoires
% usage
0
5
10
15
20
25
30
35
40
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
LG3 tadpole thymus
LG3 tadpole spleen
LG15 tadpole thymus
LG15 tadpole spleen
F.
adult Vβ repertoires
% usage
LG3 adult thymus
LG3 adult spleen
LG15 adult thymus
LG15 adult spleen

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Chapter 6

How to be cold-blooded: A theoretical perspective on the challenges temperature poses to T cell responses in poikilotherms

6.0 How to be cold-blooded: A theoretical perspective on the challenges temperature poses to T cell responses in poikilotherms

6.1 Introduction

The adaptive immune system is thought to have evolved rapidly ~ 500 mya at the advent of jawed vertebrates (reviewed by [1]). Unlike innate immunity, which is reliant on the recognition of highly conserved pathogen components, adaptive immunity is characterized by the production of two types of extremely variable receptors: immunoglobulins (Ig) and T cell receptors (TCR) (as reviewed in [1]). The adaptive immune system first evolved in a poikilothermic vertebrate (poikilotherm), and presumably provided these animals with a selective advantage in responding to pathogens in the environment [1, 2]. However, the B and T cell responses of poikilothermic vertebrates (poikilotherms) are generally not as vigorous as those of mice or humans (reviewed in [3] and [4]). This has been attributed to the lack of lymph nodes and germinal centres in poikilotherms, the argument being that cells of the immune system cannot associate with each other efficiently without being localized to these structures. Here, I propose that rather than being a consequence of not possessing evolutionary innovations present in mammals and in birds, that the "less robust" B and T cell responses of many poikilothermic vertebrates stem from a need for immune function and self tolerance throughout a range of temperatures. Since the body temperature of these organisms fluctuates with the ambient temperature, this would necessitate that the system functions over a range of temperatures.

Here I will specifically deal with the effect of ambient temperature changes on T cell selection, function, tolerance and maintenance.

6.2 The Hypothesis

Here I make the theoretical argument that the "less robust" immune responses of many poikilothermic vertebrates result from a need for immune function over temperature changes they encounter in their environment. Based upon our growing knowledge of T cell selection and activation in mice, it seems clear that T cell function should be especially affected by changes in ambient temperature. The "strength" of the interaction of TCR with peptide-MHC complexes changes significantly with temperature, and there are indications that the fine specificity of the TCR may change over temperature ranges commonly encountered by many poikilothermic vertebrates.

This has many implications for repertoire selection and peripheral repertoire size as well as the prevention of autoimmunity and the specificity of memory in poikilothermic vertebrates. There are indications that poikilothermic creatures have adaptations that allow them to modulate their immune responsiveness with temperature changes. Previous studies have shown that changes in immune responsiveness can be partially attributed to changes in membrane fluidity at low temperature which are compensated for by the animal modulating the lipid make-up of the membrane to increase the fluidity and restore T cell responsiveness. This is definitely part of the explanation, as noted by previous researchers, but cannot fully account for all of the observations. Recent advances in experimental technology should allow us to study T cell specificity and function in poikilothermic vertebrates.

Intriguingly hibernating mammals which also undergo huge variations in body temperature (heterotherms) also lose T cell function at low temperature due to T cell death. These cells are replaced by a new wave of T cells each spring.

Have poikilothermic vertebrates evolved mechanisms to deal with the effects of temperature shifts?

Is the "less robust" immune response of poikilothermic vertebrates due to a lack of lymph nodes and germinal centers or an adaptation to the realities of living at different temperatures?

6.3 Adaptive immune responses in poikilotherms

6.3.1 The nature of the response

The immune response of modern poikilotherms has been described as being more limited than that of homeotherms (meaning mice, humans, and birds) [3, 4]. The defining characteristics of adaptive immunity are diversity, specificity and memory. All of these factors have been described in the literature as being more limited in poikilotherms than in homeotherms (as reviewed in [3]).

The antibody diversity in poikilotherms is more limited than in mammals and the response slower [5]. Although significant germline diversity exists in poikilotherms, and is augmented by somatic hypermutation and receptor editing, only a slight affinity maturation is observed in secondary responses ([6, 7] and reviewed in [3] and [8]). This has been attributed to an inefficient selection of B cell clones, due to the absence of germinal centers in poikilotherms, a theory now supported by results from *Xenopus* [3, 4, 6].

T cell responses in poikilothermic vertebrates tend to be slower than in homeotherms due in part to slower cell division rates. T cell responses are compromised to a greater extent during temperature shifts than B cell responses, a fact mainly attributed to the decreased membrane fluidity at low temperatures [9].

6.3.1.1 Memory in poikilotherms

It has been suggested that a true memory response only developed in homeotherms [4, 10]. While it is true that the efficiency of the memory response is significantly higher in homeotherms, it is intriguing to note that frog memory T cells survive through metamorphosis while naïve cells appear to be systematically destroyed ([11] and reviewed in [12]). Furthermore, in catfish, memory T cells are not inhibited to the same extent as naïve cells by a decrease in ambient temperature [9]. This implies that memory is important in these animals.

6.3.2 Adaptive responses in poikilotherms: worse or just different?

Lymph nodes and germinal centers are only present in warm-blooded vertebrates. T and B cells in these centers interact with antigen presenting cells and with each other. The lack of these centers in poikilothermic vertebrates is thought to lead to a lower probability of T cells interacting with specialized antigen presenting cells in the body and thus a less efficient immune response. Are poikilothermic vertebrates merely unlucky?

Did an evolutionary breakthrough occur after the divergence of homeotherms and poikilotherms providing homeotherms with a more robust adaptive response by providing them with lymph nodes and germinal centers?

This is a fairly mammal-centric view of the situation.

6.3.2.1 Selection pressures on the response should differ between poikilothermic and warm-blooded animals

Shifts in body temperature should be a major selective force on the immune system. The high body temperature of homeotherms provides conditions for rapid pathogen growth therefore necessitating a rapid immune response especially when a cellular immune response is appropriate. The uniformity of temperature enjoyed by homeotherms affords them the opportunity to optimize their immune response to work in a very narrow physiological window. Poikilotherms undergo temperature changes, which aside from any predicted effects on the kinetics of receptor/ligand interactions needed for T cell activation, also drastically alters the ability of pathogens to replicate. Temperature changes provide profound challenges to adaptive immunity. The immune response of poikilotherms must be plastic enough to sense and respond to these changing conditions.

Even in this simplified description of the challenges faced by cold and warm-blooded animals, it is clear that the mandate/focus of each may differ significantly.

It is important to consider that in directly comparing the immune function of poikilotherms to that of homeotherms, we may be ignoring fundamental differences in the immune challenges they face.

6.3.2.2 Mammals cannot be defined by only mice and humans

Homeotherms are considered to have more robust adaptive immune responses than that of poikilotherms, but there are exceptions to this maxim. Little is known about the immune systems of mammals with different life cycles such as hibernating mammals (heterotherms) which also face the fluctuations in body temperature. In assuming that the adaptive immune response of poikilotherms is less robust than that of homeotherms we treat the immune response of obligate homeotherms such as mice and humans as some *de facto* norm. However, one cannot even generalize the immune functions of all mammals. The importance of lymph nodes and germinal centers in mammals, which undergo substantial changes in body temperature are not clear. Little is know about germinal centres and lymph nodes in hibernating mammals. We also do not take into account the range of temperatures over which poikilotherms can mount adaptive immune responses. At 25°C the frog or fish may produce a more vigorous adaptive immune response than an obligate homeotherm, such as a mouse or a human.

It is becoming increasingly apparent that the TCR/ peptide-MHC interactions required for T cell selection, activation and peripheral survival are temperature dependent [13-16]. Here I will revisit the issue of T cell responses of poikilotherms and specifically address the issue of how function changes with temperature and how these changes may occur. I propose experiments which should help forge a more satisfying understanding of these issues. Finally, I discuss how ideas pertain to adaptive immunity in hibernating mammals.

Before presenting what is known about T cells in poikilothermic animals it is necessary to provide a context for this information in terms of current theories of T cell selection, activation and tolerance. It is not my aim to review all aspects of current models of selection but rather to indicate relevant variables, which may affect T cell signaling in poikilotherms. Several recent reviews have dealt with the extensive body of evidence upon which current models are based [17-19, 20, 41].

6.4 Models of T cell selection: variables which may affect T cell signaling in poikilotherms

6.4.1 Antagonists and agonists

The nature of T cell selection and activation is still being hotly debated, even in mammalian systems where it has been most studied [21]. It has been clear for quite some time that peptide-MHC complexes interacting with a single TCR can be divided into three main categories. Agonist peptides bound to MHC lead to full activation of the T cell. Peptides, which are partial agonists result in only a partial activation of the T cell, and antagonists can actually block activation by agonists. The difference between an agonist and antagonist can be as little as a single amino acid change in the peptide held in the MHC groove (reviewed in [17]). Several types of models have been put forth to explain how T cells can differentiate between peptides which act as agonists, weak- agonists or antagonists [21].

6.4.1.1 Conformational models

Initially, it was thought that the binding of a TCR to an agonist or antagonist would differ considerably leading to models predicting differences in conformation upon binding. Crystallography data shows a remarkable degree of structural similarity between interactions with agonists and antagonists, therefore conformational differences in binding are unlikely to be responsible for the discrimination between agonists and antagonists [17, 22, 23].

6.4.1.2 Kinetic models

Kinetic models predict the different effects of altered peptide ligands is dependent on some aspect of the TCRs interaction with peptide-MHC.

Early models proposed that peptide variants, which had a higher affinity interaction with the TCR, were better activators [24]. Now, after many more measurements using different TCRs, the potency of an MHC ligand (peptide) to activate a T cell has been shown to correlate more with its off rate or the half life of the interaction than with its equilibrium binding affinity (k_d) [16]. Slower dissociation rates are associated with T cell responses [13, 22, 25-29], but this is not a steadfast rule [27, 30]. This pattern is apparent for interactions with both class I [13] and class II MHC [26]. The difference in dissociation rates of altered peptide ligands can differ by as little as 10 fold (see [31]), between an agonist and an antagonist [32].

There is growing evidence that the positive or negative selection of T cells in the thymus is correlated with the longevity of TCR-peptide-MHC complexes [33]. Several models postulate that T cell activation is dependent on short lived signaling intermediates. The 'serial triggering model' proposes that the internalization of activated TCRs allows T cells to quantitate the amount of stimulation they have received [34-37]. The length of time, which the signals are given through the TCR, may play a role in the extent of activation (reviewed in [38]). Stimulation with agonists is thought to result in an optimal amount of signaling intermediates or internalized TCRs. TCR interactions resulting in much faster or slower off rates do not result in a build-up of sufficient signaling intermediates for full activation, Longer interaction may give strong signals but results in less signal accumulation during a set time of interaction.

In addition to the kinetics of the TCR/ peptide-MHC interaction, the clustering of receptors on the surface of the T cell and APC is important, and increases the avidity of the interaction and local concentration of signaling molecules. When a T cell makes contact with an APC carrying an agonist peptide, a characteristic bulls-eye pattern forms which is enriched in specific membrane associated molecules. This structure has been dubbed the immune synapse [39-42]. Longer adhesion molecules (most notably, LFA-1s interaction with ICAM-1) on the cells interact first pulling the cells together. As the cells come together, longer molecules in addition to some cytoskeleton associated proteins such as talin move to the outside of the bulls-eye. Other interactions involving shorter molecules such as peptide-MHC binding to CD4 or CD8 and to the TCR, occur in the center. Signaling components associated with the co-receptors and CD3 complex are enriched at the synapse (reviewed in [39]). Although the affinity of the TCR/ peptide-MHC interaction is quite low (0.1 to 500μM), the co-receptor and adhesion molecules associated with the synapse hold the cells together strengthening the interaction.

The formation of a synapse is fairly well correlated to the dissociation rate (off rate) of the TCR/ peptide-MHC interaction (reviewed [43]). However, recent results suggest that synapse formation is not needed in all cases for T cell activation [44].

Synapse based models claim that membrane structure and fluidity are central to T cell signaling, for, the TCR heterodimer and the co-receptors as well as the CD3 complex must move laterally in the membrane to form an immune synapse. The TCR complex and many associated signaling related proteins are associated with cholesterol rich membrane domains sometimes referred to as RAFTs [45-48]. RAFTs are thought to concentrate signaling molecules needed for T cell activation.

All of these issues have implications for T cell education, activation and survival in poikilothermic animals (poikilotherms) because the strength of TCR signaling is intimately linked to the kinetics of TCR peptide-MHC interaction as well as the membrane fluidity, both of which are affected by temperature.

6.4.2 Repertoire selection in poikilothermic animals: the quandary of selecting and maintaining T cells in a changing situation

6.4.2.1 The Grossman & Singer model

A simplified model of T cell selection typified by the Grossman & Singer model remains the model of choice for textbooks and reviews [49].

The Grossman and Singer model outlines selection in the thymus in relation to signal intensity. Although it does not encompass all that is known about the intricacies of T cell selection, it provides a good conceptual starting point. In figure 6.1A, thymocytes that receive a signal above the maintenance threshold (V) are given a survival signal while those below this threshold die of neglect. However, cells, which receive a signal above the activation threshold (A), are negatively selected (deleted) because they may pose an autoimmunity risk [49]. Signal intensity is used to refer to the amount of signal that is received through the TCR and correlates experimentally to selection outcomes [50, 51]. This sort of model has been experimentally validated by Savage and Davis [52] who have reported that cells which successfully enter the periphery after positive and negative selection in the thymus possess TCRs with off rates which fall into "a distinct kinetic window", excluding those with either very high or very low off rates. In addition, the amounts of signaling intermediates have been quantified. In cases of low or no signaling there is no positive selection whereas much higher amounts of signaling intermediates result in negative selection [50, 51]. Although the requirements for, and events of TCR signaling remain controversial, it is apparent that the strength of the signal delivered to the T cell is a determining factor in positive and negative selection as well as peripheral T cell activation and survival.

6.4.2.1.1 Shifts that increase the intensity of signaling through the TCR

Assuming the existence of a "window of selection", temperature changes could dramatically change the size of the peripheral repertoire in poikilothermic animals. In figure 6.1B, let us consider the case where a temperature change increases the signal intensity through the TCR of T cells already in the periphery of the body. In this case the repertoire shifts up. Cells with thresholds just above the viability maintenance threshold become cells in the main portion of the viable repertoire. No other cells fill out the lower repertoire because they were deleted in the thymus during selection. More importantly, the shift will mean that there are now cells with a signal intensity in the periphery which would have been deleted in the thymus. These pose a potential autoimmunity risk for they have not been fully depleted of cells which can recognize self tissues. In mammals, naïve T cells must receive two signals to become activated. The first signal is thought to be delivered through the TCR, and the second through an interaction with an antigen presenting cell (APC). This second signal is referred to as costimulation, and not all APCs can deliver this signal. Naïve T cells receiving only the first signal, enter a state of unresponsiveness (anergy). Thus, peripheral T cells, which recognize a self-peptide without costimulation, become unresponsive (anergic). If all potentially self reactive cells in the shifted population become anergic, they are lost from the functional peripheral repertoire decreasing the variability present in the repertoire. Mammalian memory T cells however, generally do not need the same level of costimulation as naïve T cells to become activated [53-55].

6.4.2.1.2 Shifts that decrease the intensity of signaling through the TCR

In the case of a decrease in signal intensity, as in figure 6.1C, the animal loses the highest responders in the peripheral repertoire and now a portion of the repertoire drops in to the range below the viability maintenance threshold. These cells would not be strong enough responders in the thymus to survive and in the periphery probably would go into a type of anergy due to an inability to interact with self MHC molecules, and be lost from the functional peripheral repertoire. T cell signaling resulting from low affinity interactions with self-MHC in the periphery is needed for the survival of naïve T cells in mice [56-59]. The loss of interactions with MHC by some of the T cells in the shifted population may lead to the death of T cells which can no longer interact with self MHC molecules.

Both increases and decreases in signal intensity are therefore predicted to decrease the active, usable peripheral T cell repertoire. This reduction is likely to affect species with a low total number of T cells more than larger animals because the size of the peripheral repertoire is so much smaller in the smaller animal (see chapter 7 for a further discussion of this idea).

6.5 Temperature changes should affect T cell selection and function

Temperature dependent changes in the kinetics of receptor ligand interactions (off rates), membrane fluidity, as well as related changes in signaling, and in membrane receptor levels all have the potential to modify the intensity of signal which a T cell receives. Here I discuss recent studies in homeotherms which suggest which of these factors are likely to be important in the adaptation of the system.

6.5.1 The binding kinetics of the TCR peptide MHC-interaction change with temperature

There is ample evidence that the affinity and the on/off rate of the TCR interaction with MHC/peptide complexes changes with temperature. This is due to thermal effects on protein-protein interaction and is independent of temperature dependent changes in membrane fluidity.

Several studies have suggested that the T cell receptor, independent of the CD3 complex or co-receptor shows temperature dependent kinetics when binding MHC/peptide complexes. These studies employ either the binding of immobilized TCR protein to a soluble form of peptide-MHC or *vice versa* (plasmon resonance) or the binding of peptide-MHC complexes which have been assembled into synthetic tetramers, to TCRs on intact cells [14, 16, 26, 60-63].

6.5.1.1 Methods used to study TCR peptide-MHC binding

In plasmon resonance (BIAcore) studies, one molecule is covalently bound to a specially coated surface and a solution containing the ligand is passed over this surface with the bound receptor. Binding of the molecule to its ligand results in a change in the resonance state of gold electrons on the surface of the apparatus, allowing the binding kinetics of the reaction to be measured [31]. BIAcore measurements of the binding of soluble MHC/peptide complexes to immobilized TCR molecules show that the rate constants increase with temperature. The binding kinetics of peptide-MHC tetramers to intact T cell hybridomas confirms the affinities measured by the fully cell free BIAcore system [60]. The binding affinity increased as the temperature was lowered from 37°C [13, 24, 64]. Other studies employing immobilized MHC/peptide complexes measured the binding kinetics of soluble TCR. Binding of soluble TCR to a specific peptide-MHC complex (agonist) increases markedly when the assay temperature is shifted from 25 to 37°C whereas the binding to the same MHC carrying a slightly modified peptide (a weak agonist) was only slightly changed [65].

Most BIAcore studies are carried out at 25°C. When these results are compared to those done at 37°C, the off rates generally become 10 to 20 times faster [64, 66, 67]. Over smaller changes in temperature (i.e. 5°C) one can expect off rates to be at least 5 times faster. This is unlikely to be an artefact of the BIAcore method, for studies of the binding of soluble peptide-MHC complexes to live T cells are also complicated by differences in binding kinetics at 4, 25 or 37°C [16, 68].

6.5.1.2 Off rates and half-lives of TCR peptide MHC complexes change significantly with 5-12°C changes in temperature

Off-rates for agonists tend to fall within the range of 0.5 $\sec^{-1}$ to 0.01 $\sec^{-1}$ for a weak agonist and a strong agonist, respectively [31]. The half-life of the complex ($t_{1/2}$) is calculated by the formula: $t_{1/2} = \ln 2 / k_{off}$. Therefore, the half-lives of a weak agonist and a strong agonist are 1 and 60 sec, respectively. If the off-rate changes between 5 and 20 times with a temperature change of 5 to 10°C, then we expect changes in the half-lives of these complexes. In some systems a 10-fold difference in dissociation rates can differentiate an agonist from an antagonist. Such changes could lead to an antagonist becoming a weak to intermediate agonist, and weak agonists becoming strong agonists or antagonists depending on the direction of the temperature shift. Therefore, temperature dependent changes in TCR binding kinetics are expected to play a significant role in determining the intensity of T cell signaling in poikilothermic animals.

6.5.1.3 Temperature changes can change the fine specificity of the TCR

The binding of soluble peptide-MHC tetramers to TCRs on cells changes markedly with temperature [16]. Furthermore, the fine specificity of the TCR binding changes with temperature. Peptide variants which bind a TCR poorly at 37°C can often bind at lower temperatures, effectively changing the fine specificity of the TCR [16, 68]. This could be profoundly important for poikilotherms. It is often argued that somatic hypermutation does not occur in TCR due to the dangers of changing the fine specificity of the TCR and potential autoimmunity which could result [69]. In poikilotherms a change in specificity may occur with changes in temperature and in an environment which might preclude proper selection of this new specificity. The need for costimulation to activate naïve T cells could prevent autoimmunity leading to the anergy of self reactive T cells in the periphery. However, as already discussed, this would come at the price of losing a proportion of the peripheral repertoire and limit availability of TCR variability in the periphery. Memory T cells, may become dangerous if their fine specificity changes because they are thought to have less stringent requirements for activation and are less likely to become tolerized in the periphery [53-55]. Are the slower and lower T cell dependent responses observed in ectotherms partially a consequence of this potential danger? Are there different requirements for secondary responses in poikilothermic vertebrates?

6.5.1.3 Can we predict that the potential reactivities of poikilothermic TCRs will mirror the results in homeotherms?

Although there has not been an exhaustive cloning of TCR segments in most species, it is apparent that poikilothermic vertebrates have maintained quite a variable genomic repertoire of TCR segments. In *Xenopus*, TCR β segments a similar level of genomic variability is maintained in *Xenopus* as compared to that of mice and humans (chapter 2). This variability may provide enough diversity to ensure a population of T cells with receptors which could work over a range of temperatures. TCR sequences in poikilotherms and homeotherms are generally well conserved, maintaining the residues, which make up the immunoglobulin fold and other TCR specific residues (as reviewed in [70]). In addition, MHC class I and II molecules are quite similar to those of mammals. This suggests that the temperature dependent kinetics measured in homeothermic TCRs will also hold for TCRs in poikilothermic vertebrates. General comparisons of homology can be misleading however. A study of an Ig heavy chain from an antarctic fish, *Trematomus bernacchii*, showed an aa identity of between 53 and 61% with that of other bony fish. However, an insertion at in the hinge region is hypothesized to provide extra flexibility to the receptor, a common adaptation strategy in the proteins of organisms that live at low temperatures [71].

If molecular flexibility is a common adaptation to low temperature we must note that the TCR interface is much more flexible than that of immunoglobulins [72, 73]. While the measured homologies between TCR β chains seem straightforward and profound, the flexibility of these chains is difficult to predict. Only functional data will help us determine if TCRs from poikilothermic vertebrates function differently from those of homeotherms.

6.5.2 Membrane fluidity

6.5.2.1 Membrane fluidity and the immune system

Because formation of an immune synapse (see section 6.4.1.2) requires lateral movement of proteins in the membrane, the fluidity of the membrane is a central issue in T cell activity in poikilotherms. The effects of temperature on the immune system has been most thoroughly addressed in fish (reviewed in [74]). The optimum temperature for immune function varies with the species of fish. Warmwater fish have optimal immune responses at 20-30°C and cold-water fish at 10-15°C [75]. When the temperature is lowered (or in some cases, when it is raised) there is a range of temperature at which immune function continues but is compromised [76]. Temperatures lower or higher than this result in a loss of immune function and are termed, non-permissive [77]. T cell responses are preferentially affected by temperature changes while B cells can still respond to the B cell mitogen LPS and APCs can still take up and process antigen as well as secrete the cytokine, IL-1 [78-81].

The loss of T cell activity at low temperatures has been attributed to a reduction in membrane fluidity in T cells at non-permissive temperatures [82-86]. This conclusion is supported by several observations. First, proliferative responses to the T cell mitogen ConA can be partially regained by treating cells with a monosaturated fatty acid, oleic acid also resulting in an oleic acid dependent increase in membrane fluidity [85]. Second, unlike B cells, T cells have a low level of the enzyme (stearyl-co-enzyme-A-desaturase) which converts stearic acid to oleic acid [85]. Third, fish, which were kept at a non-permissive temperature for a few weeks, regained T cell function [9]. It is well known that fish, over a period of hours to weeks compensate for temperature induced differences in membrane fluidity by changing the lipid to sterol composition of membranes in a process called "homeoviscous adaptation" [86-92]). B cells from channel catfish took 3 weeks to undergo homeoviscous adaptation to 17°C and 5 weeks for 12°C [92], whereas T cells took a minimum of 5 weeks [86].

6.5.2.2 Why is homeoviscous adaptation so "slow" in lymphocytes?

The length of time needed for homeoviscous adaptation in lymphocytes is somewhat surprising when compared with other tissue types. In fish, the speed and extent of homeoviscous adaptation differs according to the tissue type. In trout liver, kidney and gill tissue, a significant change in the ratio of cholesterol to phospholipids was seen upon shifting the temperature from 20°C to 5°C. Conversely, erythrocytes did not change the fluidity of their membranes [88, 93]. Similarly, trout hepatocytes underwent significant homeoviscous adaptation over a 5 hour period and the inner hemilayer of the membrane was preferentially modified during this short term exposure [94]. Significant changes in the ratio of membrane components were also observed upon temperature change in brain tissue from rats, birds, fish and arctic fish, but little difference was observed in the functional ordering of the membranes [95]. This similarity in membrane ordering suggests membrane fluidity serves a function and is actively regulated in a tissue specific way.

Thus, lymphocytes while differentially regulated are not preferentially modified or continuously modified by mechanisms, which can precisely modulate membrane fluidity with temperature in other tissue types.

6.5.2.3 RAFTs

It is unclear if cholesterol rich membrane domains (RAFTs), which in homeotherms seem to enrich T cell signaling components, are important in poikilothermic animals. Cholesterol is one of the components that is reduced at low temperatures in trout membranes [88]. The reduced responsiveness of T cells at low temperature could also be attributed to the lower amount of cholesterol in the membrane due to homeoviscous adaption. In mammals, cholesterol rich membrane microdomains called RAFTs are thought to be important for concentrating TCR and signaling molecules [46, 96]. However, the presence of cholesterol rich RAFTs is certainly not the only important factor. Treatment of a human T cell line (Jurkat) with poly-unsaturated fatty acids (PUFAs) leads to the loss of molecules involved in TCR signaling (the Src kinases Lck and LAT) from RAFTs [97] and is used clinically to suppress the activation of human T cells. Also, the depletion of cholesterol from the membranes of mouse T cells leads not to a lack of all signaling but to a transient phosphorylation of molecules involved in the signaling cascade (CD3ζ and ZAP-70) [45]. This suggests a specific control of function and membrane fluidity/rigidity in different cell types, rather than a general interference caused by a disruption of RAFTs by the removal of cholesterol.

When considered in light of the implications of temperature change predicted by the Grossman and Singer model, low temperature induced anergy is interesting. One cannot contest the existence of a low temperature anergy effect on T cells, but, but because signal intensities would change at these different temperatures, how would homeoviscous adaptation work? Anergy at low temperatures may prevent memory T cells from being formed at lower temperatures, but existing memory cells remain active, as secondary T dependent responses are not affected [9, 74]. Since homeoviscous adaptation modifies lymphocytes in animals that have been kept at a sustained low temperature, how do these cells safely move from anergy into activity? Is the length of time required for homeoviscous adaptation really due to the time required to select an entirely new repertoire of cells, or are existing T cells being modified? Over smaller temperature changes is membrane fluidity controlled to set, and perhaps maintain, the signaling threshold of T and B cells?

Regulation of membrane fluidity has also been suggested to play a role in tuning of T cell thresholds during the development of murine T cells [98]. Membrane fluidity is likely to be a major level of regulation of T cell responses in poikilothermic animals, but it is increasingly clear that it is not the only level of regulation. Aggregation of surface receptors (capping) occurs on fish T cells even at non-permissive temperatures and occurs more efficiently on T cells than on B cells. The membrane at non-permissive temperatures is evidently fluid enough to allow the lateral movement of proteins in the membrane required for capping [92, 99]. As we will see below, signaling can also reverse low temperature induced anergy in some situations.

6.5.3 Temperature dependent effects on signaling

The inhibition of channel catfish T cells at non-permissive temperatures can be overcome by treatment with phorbol ester or calcium ionophores [100]. Phorbol ester and calcium ionophores activate the TCR signaling pathway downstream of the TCR by increasing cytoplasmic free calcium and activating PKC, a serine threonine kinase (as reviewed in [101]). Thus, although signaling can take place, it does not normally occur through the TCR at non-permissive temperatures in naïve T cells.

In channel catfish, calcium ionophore or phorbol ester treatment of primary T cells results in T cell lines, which are, immortalized [102, 103]. These cells produce soluble factors (cytokines?) which can restore the responsiveness of naïve T cells to ConA and restore primary responses to T dependent antigens in fish raised at non-permissive temperatures [74]. Stimulation by these supernatants appears to be through the Jak/Stat pathway via tyrosine phosphorylation of a STAT like molecule [104]. This suggests that despite the rigidity of T cell membranes at non-permissive temperatures that signaling and proliferation is possible. T cell responsiveness may therefore be controlled at the level of signaling.

Thus, primary cells can be activated from their anergic state at non-permissive temperatures but memory cells are not temperature sensitive [9, 74]. Consequently, the production of cytokines by memory T cells at non-permissive temperatures, has the potential to break the anergy of naïve T cells at low temperatures. It would be intriguing to know if this does happen *in vivo*.

As discussed above, capping (a polar aggregation of receptors on the cell surface) is also affected by changes in membrane fluidity in acclimatized T cells, even at non-permissive temperatures [99]. This is interesting because in mice, the formation of a T cell synapse or B cell capping are both dependent on actin polymerization in the lymphocyte but not in the APC [105]. In mice, cap formation on B and T cells is dependent on VAV, a guanine exchange factor, the Rho family kinase, Rac1, and the Wiscott Aldrich syndrome protein (WASP) (reviewed in [106, 107]. Furthermore, VAV and Rac1 are necessary for the movement of lipid RAFTs into the T cell synapse of human (Jurkat) T cells [108]. T cell signaling may therefore play an important role in altering the fluidity of the membrane and the actin dependent movement of molecules and membrane domains.

6.5.4 Modulation of surface receptor density

Another possible way to control the level of signaling in T cells is the manipulation of the number of surface receptors or signaling molecules [92]. Experimental manipulation of the surface density of MHC molecules in mammalian cells influences T cell selection [109] and activation[110].

There are few studies of the temperature dependant T cell modulation of surface receptors involved in function. Rodrigues et al [111] have reported that there is a decrease in surface MHC expression on catfish antigen presenting cells (APCs) when these cells were shifted to lower temperatures. This reduction in class I MHC expression was due to a reduction in the transcription of β-2 microglobulin. β-2 microglobulin protein is required for surface expression of class I MHC molecules. At 12°C, a non-permissive temperature, a total down regulation of β-2 microglobulin transcription was observed but transcription began again 3 days later. At 4°C, there was a full, lasting loss of β-2 microglobulin expression which could only be restored by shifting the temperature back to 12°C. Thus, the surface density of MHC class I molecules is modulated with temperature in catfish. There are no corresponding studies detailing what effect, if any, temperature changes have on MHC class II expression.

Modulation of the density of surface molecules is a common adaptation in poikilothermic animals, as typified by the example of cold-temperature adaptation of the adrenergic receptor/adenyl cyclase signal transduction pathway in trout hepatocytes. cAMP production is slower at 5°C than at 20°C but two times as much cAMP is produced. There is a 50% compensation of lipid bilayer order but increasing the membrane fluidity by chemical means actually decreases cAMP production. The increased cAMP production was attributed to the expression of a 1.8 fold higher number of beta-adrenergic receptors on the surface of 5°C acclimatized cells. [87] The authors argue, convincingly, that the modulation of surface receptor number is more important than membrane fluidity in trout hepatocytes.

In mice, interactions with ligands which are below the threshold for positive selection appear to play a role in setting the activation threshold of developing T cells by modifying the amount negative regulators of T cell signaling such as, CD2 or CD5 on the cell surface [112]. This process has been dubbed dynamic tuning. CD5 is thought to play role in reducing T cell receptor signaling, as knockout mice have hyperesponsive T cells [113], and that CD5 expression on thymocytes correlates well with the avidity of selecting ligand and developmental changes in T cell reactivity [114]. Dynamic tuning would raise the threshold of developing thymocytes as they move through the thymus based on the avidity of the selecting ligand.

The surface density of the CD8 co-receptor has also been found to influence the response to altered peptide ligands presumably by increasing the avidity of the interaction and slowing the off rate [115, 116]. It has been suggested that modulation of CD8 surface expression can help many T cells attain a strength of interaction which allows them to mature and enter the periphery [116].

Changes in the surface density of molecules involved in T cell adhesion or signaling clearly do affect the outcome of the T cells interaction with the APC in mammals. Changes in surface receptor density are used by many poikilotherms for temperature compensation. Modulation of the density of surface molecules could therefore conceivably be used in poikilotherms to change the activation thresholds as temperature changes TCR kinetics or to decrease the responsiveness of T cells at non-permissive temperatures.

6.6 Discussion

6.6.1 Practical relevance

Temperatures as low as 12°C are commonly found in commercial fish ponds and the temperature seldom stays constant over the 5 weeks that would be needed for complete homeoviscous adaptation [92]. Temperatures can fluctuate considerably over the course of even a single day with the weather or with shifts in water depth for aquatic poikilotherms [117]. This has been used to argue that the catfish adaptive immune response is likely to be constantly constrained by incomplete homeoviscous adaptation [92]. If this is the case, we can question whether T cell mediated immune responses play an important role in the immune responses of poikilothermic vertebrates that commonly undergo such temperature shifts. It is important to remember that only naïve T cells are temperature sensitive [9]. If T cell function and T cell memory was not important for the survival of these animals, would this distinction be evolutionarily maintained? This argues for the importance of T cells.

While these issues may seem academic, they have important practical relevance. Many millions of fish are immunized each year. If temperature plays a role in the production of memory for antigen, then temperature manipulation during immunization and boosting (reimmunization) may be necessary. If the survival of memory T cells is decreased, or their specificity changes at low temperature, or over a range of temperatures, then effective immunization may prove almost impossible. Determining the primary mechanisms by which T cell activation is mediated is vital for our understanding of vaccination and immunotherapy in both poikilotherms and homeotherms. Knowing which factors are involved here will clarify poikilothermic immunity. An understanding of how poikilotherms manipulate membrane fluidity, avidity or signaling might help us make sense of what factors are important in the homeotherms, which, most likely, represent a very specialized case. Comparisons between tropical fish and homeotherms which maintain a constant body temperature, with poikilotherms (and heterotherms, see next section), which live over greater temperature ranges may prove useful. Perhaps such studies can illuminate those adaptations that are due to, or made possible by, life at a relatively constant temperature.

T cell function in poikilothermic vertebrates is likely to be a modulated by a combination of factors but over small temperature changes the kinetics of receptor interaction or some factor in the signaling pathway is likely to be more important than membrane fluidity.

6.6.2 How is T cell memory maintained in poikilothermic animals?

Studies of T cell selection and activation in mammals have strongly suggested that the ability of T cells to differentiate between self and non-self peptides is a question of the signaling threshold and the specificity of the T cell. Selection is thought to remove potentially self-reactive cells by selecting those T cells with receptors that fall into the window of signaling between being "too strong" and or "too weak". Only those that are "just right" are allowed into the periphery of the body. This is fine if the strength of signaling is kept stable so that T cells in the periphery can continue to differentiate self from non-self. But, in fish or frogs, T cell interactions may not remain stable. As we have seen, this may lead to anergy in the naïve cell population, perhaps protecting the animal from autoimmunity. Presumably, if kept at a new set temperature for long enough, cells can regain their bearings on what constitutes self and non-self. This has indisputable implications on T cell dependent responses and the size and variability of the usable peripheral repertoire of T cells in poikilotherms.

The more pressing potential problem is T cell memory over a range of temperatures. Memory T cells in fish and mice appear not to be anergized by shifts to lower temperatures, which are non-permissive for naïve T cells [9, 118]. If the fine specificity of these memory cells can change with temperature, as suggested by studies on mammalian cells, these T cells may be able to recognize self-peptides. Do poikilothermic memory T cells require costimulation for memory responses? Perhaps this is part of the reason that memory responses in poikilothermic animals are considered not as robust as those of most warm-blooded animals.

These ideas can now be tested using the channel catfish model where T cell lines are available [74, 119, 120]. T cell receptors and MHC molecules could be cloned and their binding kinetics tested using plasmon resonance (BIAcore) techniques at different temperatures. Because there are currently no markers specific for memory T cells in poikilotherms, it is difficult to address if the memory T cell population undergoes a more complete homeoviscous adaptation in relation to the naïve T cell population. However, because the memory T cell response is less temperature dependent, we can hypothesize that memory T cells have more fluid membranes. Staining T cells with fluorescently labelled MHC-peptide tetramers may help determine the antigen specificities of T cells and serve to purify antigen specific memory T cells through fluorescence activated cell sorting. The injection of these cells into mice could lead to the production of monoclonal antibodies that recognize memory specific markers on such cells.

6.6.3 NK receptor affinity may also change markedly with temperature

Many of the same issues are also relevant to the function of NK cells in poikilotherms. NK receptors have been cloned in poikilotherms (i.e. NITR; novel immune type receptors) and cells with an NK phenotype are found [121-126]. If NK cells in poikilotherms use a system of balanced inhibitory and stimulatory receptors like those in mammals and chickens, then a change in the affinity or kinetics of signaling through these interactions could have disastrous consequences. How would such a balance be achieved and maintained?

A system of education or selection for NK cells is implied by the slightly differing combinations and surface densities of inhibitory and activating NK receptors on NK cell populations in mice and humans (reviewed in [127, 128]). Furthermore, class I knockout mice and β_2-microglobulin (β_2m) knockout mice possess cells with NK markers but which do not attack self tissues lacking class I [129, 130]. The global effects of temperature on NK cell responsiveness have not been studied in poikilotherms or in heterotherms. However, in mice it is clear that the NK cell receptor, NKG2D, has a different affinity for its ligands: H60 and RAE1 [131]. This interaction is extremely temperature dependent, with binding to H60 being much more temperature dependent than that of RAE1. Thus it has been suggested that this temperature dependence is such that a temperature increase such as fever could drastically change the ratio of NKG2D binding to RAE1 and H60. Although NK receptors are generally not very well conserved even between mice and humans, NKG2D homologues are found in both species [132, 133]. The NK receptors that have been cloned in poikilotherms are likely to show some form of adaptation to the changing temperatures encountered in these species. [122, 125, 126]. NK receptors have not been cloned from heterotherms.

6.6.4 Is temperature an issue in T cell immunity in heterotherms as well?

Although heterotherms (hibernating mammals) do not undergo the same process of homeoviscous adaptation as poikilothermic vertebrates do, we still see the same seasonal patterns of T cell death in winter and production of a new wave of T cells in the spring (seasonal histogenesis) [134-143]. Is this a homologous or analogous process?

Studies of the function of mouse T cells at 27°C showed that T cells that were stimulated through the TCR at this low temperature died, but they could be rescued by increasing the fluidity of the membrane with oleic acid [144]. Thus the same issue of membrane fluidity seems to apply to T cells in mice. Intriguingly, low temperature (27°C) only suppressed the activity of naïve T cells while memory T cells and B cells could still function. Naïve cells could be rescued by the addition of oleic acid or the cytokines IL-2 or IL-4. IL-1 could not rescue cells [118]. Therefore, the response of mammalian T cells to low temperature is quite similar to that seen in fish. One possible conclusion is that, for yet unknown reasons, T cell function is undesirable at low temperatures.

6.6.5 Is the adaptive immune system "too expensive" at low temperatures?

Perhaps the adaptive immune response is too energy hungry to be maintained by animals at low temperatures. Animals tend to produce less energy at low temperatures and therefore adaptive immunity, with its "wastage" of cells in the thymus during selection, may put a strain on the animal. It would be a situation quite analogous to the down-regulation of the immune response during the so-called "fight or flight" responses by compounds such as glucocorticoids [145-147]. The rationale is that you have no need for an immune response if a bear catches you today, better to put the energy into running. Similarly, what good is an adaptive immune response if you freeze or starve to death due to a lack of energy at low temperatures? This may explain why both poikilotherms and homeotherms which undergo seasonal fluctuations in temperature, lose a great proportion of their T cell populations and which are replaced by a new population of naïve T cells upon being restored to a higher temperature.

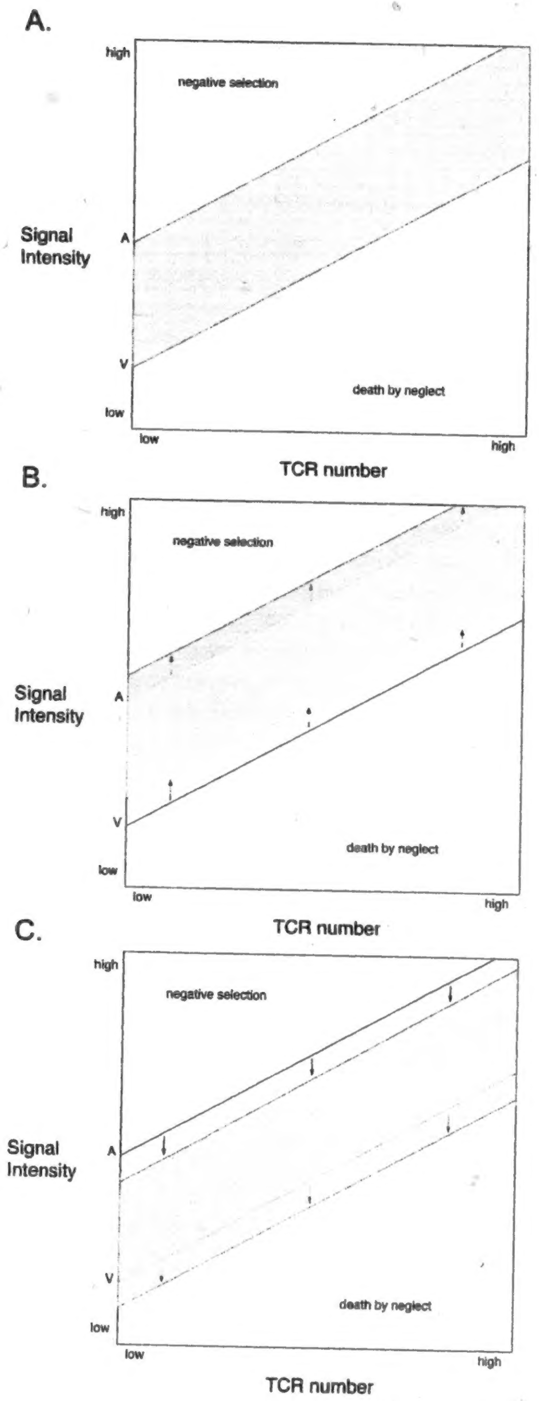
We only expect large or long-term temperature changes to be too energetically costly. The more important, and sadly least studied situation is the effects of small (< 10°C), short term fluctuations in body temperature. Our interpretation of the Grossman and Singer model suggests that short term increases or decreases in temperature will lead to a functional loss of a portion of the peripheral T cell repertoire in the absence of mechanisms which could compensate by modulating the amount of signal passed through the TCR. We have suggested several mechanisms that could compensate. There may be evolutionary compensation for life at very low or very high temperatures by selecting specific TCR segments which "perform" better over the range of temperatures the animal is exposed to. Membrane fluidity changes have been shown to modulate T and B cell activity through homeoviscous adaptation. There may be mechanisms that tune TCR sensitivity on the periphery to maintain T cell function or to break T cell anergy under certain conditions. Finally, the surface density of receptors or ligands could conceivably be changed to compensate for small temperature shifts.

A better understanding of how T cells respond to temperature changes may suggest new approaches for manipulating the reactivity of T cells *in vivo*.

Furthermore, it shows the necessity of performing experiments at physiological temperatures even when the experimental kinetics make measurements more difficult. Some conflicting results obtained from manipulating mouse or human T cells at nonphysiologic temperatures may reflect the comparison of systems in which T cells are able to modulate their reactivity because of their evolutionary heritage. It is doubtful in obligate homeotherms if all of these potential modulations are actually used under normal conditions in the animal. The study of the wonderfully complex and plastic yet precise processes involved in T cell selection, activation and memory in homeotherms and heterotherms may play a major role in defining important aspects of our situation as warm-blooded vertebrates.

Figure 6.1 Temperature dependent changes in selection

The Grossman and Singer model outlines selection in the thymus in relation to the intensity of the signal through the TCR. Signals above the maintenance threshold (V) are given a survival signal while those below this threshold die of neglect. Cells that receive a signal above the activation (A) are negatively selected and undergo apoptosis. Signal intensity refers to the amount of signal received through the TCR and correlates experimentally to selection outcomes (see text, section 6.4.2.1). In A, we see the original depiction of the Grossman and Singer model. The blue area depicts the population of T cells which pass positive selection (above V) but which are not above the activation threshold. On the x-axis we see T cell number. This reminds us that the density of TCRs on the surface of the T cell affects the amount of signal received by the cell. In B, a temperature change increases the signal intensity through the TCR of T cells already in the periphery of the body. In this case the repertoire shifts up. Cells with thresholds just above the viability maintenance threshold become cells in the main portion of the viable repertoire. No other cells will fill the lower repertoire in this case because they were deleted in the thymus during selection. More importantly, the shift will mean that there are now cells with a signal intensity in the periphery which would have been deleted in the thymus. These pose a potential autoimmunity risk for they have not been fully depleted of cells which can recognize self tissues. In C, the case of a decrease in signal intensity, is considered. The animal loses the highest responders in the peripheral repertoire as a portion of the repertoire drops into the lower part of the normal range. Another portion of the T peripheral T cell population will now receive a signal through the TCR that is below the viability maintenance threshold. These cells would not be strong enough responders in the thymus to survive selection and in the periphery probably would not be a useful portion of the repertoire due to an inability to interact with self MHC molecules, and be lost from the functional peripheral repertoire. This would theoretically lead to a form of anergy. Based on this model, both increases and decreases in temperature could affect the size of the functional peripheral T cell repertoire in poikilotherms and heterotherms.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Chapter 7

Discussion

7.0 Discussion

The work described in this thesis has given us our first detailed glimpse of what a TCR repertoire of a poikilothermic vertebrate looks like. A reagent that recognizes CD3ε, a component of the TCR signaling complex, has extended our ability to visualize all T cell subsets in *Xenopus*. We have shown that the number and the variability of TCR β segments in *Xenopus* is comparable to those of mice and humans and is greater than that found in chicken. The TCR β repertoire is also quite variable as it emerges in the tadpole just 4 days after hatching. A more in-depth study of repertoire has shown that all of the TCR Vβ segments can be used in the TCR β repertoires of both tadpoles and adults. The proportional usage of several Vβ segments changes significantly with developmental differences in class I expression or selection on the different MHC haplotypes found in LG3 and LG15 frog lines.

The thymic and peripheral repertoires of tadpoles differ significantly from those of adults. This is generally not the case in mice and humans (the exception is the deletion of entire Vβ subsets due to superantigen (sAg) stimulation) [1]. The significance of the repertoire differences between tadpoles and adults remains unclear. Developmental differences in repertoire are not seen for the same Vβ segments in animals with different MHC haplotypes. This contradicts the hypothesis that developmental differences in class I expression serve to "force" the expression or serve to prevent the expression of specific Vβ segments during tadpole or adult life. In light of the complexities of T cell selection it is perhaps not surprising that repertoire differences cannot be explained by so simple a hypothesis. Instead of primarily developmental differences, we see differences in the peripheral repertoires selected on different MHC haplotypes.

The findings presented in each part of this thesis are reviewed and future work which could further our understanding of these issues is discussed. Summaries and discussions of each topic are found at the end of each chapter, and I direct the reader there for more detail.

Here I will discuss several more far-reaching issues such as whether all class Ia molecules are equivalent from a repertoire viewpoint; why class I MHC is not expressed during tadpole life; and the issue of repertoire from the perspective of size of the animal and the temperature changes that animal encounters.

7.1 Uncharacterized populations of *Xenopus* T cells can be visualized using a polyclonal serum to CD3ε

The specificity of a polyclonal antiserum, that recognizes a conserved proline rich region in the cytoplasmic domain of CD3ε in many vertebrate species including *Xenopus* was defined in chapter 3. This reagent can be used to visualize CD3ε positive cells in cryosectioned tissues but is not well suited to preparative immunoprecipitation for protein sequencing of parts of the complex (see discussion in chapter 3). This approach is no longer necessary to clone the TCR α chain (Haire et al, accession #AF440803-AF440817), but CD3ε has not been cloned in *Xenopus*. Harsher lysis and immunoprecipitation conditions than were used in these studies may allow the CD3ε protein to be purified and sequenced.

This reagent will prove invaluable in the future in helping to define subpopulations of T or perhaps NK cells in *Xenopus*, and will allow the specificities of other lymphocyte specific antibodies to be better defined. The study of CD3ε$^+$ cells in thymectomized animals will allow us to visualize T cells which develop independently of the thymic environment. NK cells have been reported to be more numerous in thymectomized animals, and should allow the staining pattern of this reagent on NK cells to be characterized [2, 3].

7.2 The genomic repertoire of Vβ segments in *Xenopus*

7.2.1 The *laevis* and *gilli* TCR loci: first impressions

The work presented in chapter 2 represents the first exhaustive cloning of TCR β segments from a poikilothermic vertebrate. This was necessary before embarking on a study of T cell ontogeny or repertoire in *Xenopus*.

It had been hypothesized that because *Xenopus* possesses a single predominantly expressed class I locus, as is the case in chicken, that *Xenopus* like chickens would also have a minimal TCR locus due to a coevolution of the loci (Du Pasquier, personal communication). We found a surprising number of new Vβ and Jβ segments confirming that the genomic repertoire of TCR segments in *Xenopus* is not minimal as in the chicken.

9 new Vβ families were cloned. The use of LG hybrids for this study allowed the new segments as well as those segments found in a previous study [4] to be attributed to the *laevis* or *gilli* haplotypes of the hybrids. 25 Vβ segments are present on the *laevis* locus and can be grouped into 19 distinct Vβ families based upon 75% homology at the DNA level. 25 Vβ segments are found at the *gilli* locus but only 17 Vβ families are expressed. Southern analysis of haploid tadpoles showed that a Vβ3 segment is physically present on both the *laevis* and *gilli* loci but Vβ3 is never (in over 55 sequences) expressed from the *gilli* locus making it a pseudogene or a very rarely used segment. The *gilli* Vβ10 is also most likely a pseudogene but this was not formally shown. 12 new Jβ segments were also cloned in this study. These too could be attributed to the *laevis* or *gilli* locus. Thus, there are 19 and 13 Jβ segments associated with the *laevis* and *gilli* loci, respectively.

The number and variability of Vβ segments in *Xenopus* is comparable to those of mice and humans. The human TCR β locus contains 65 TCR Vβ segments, 47 of which are functional. These can be grouped into 20 families [5, 6]. There are 2 DJC clusters, each containing a single Dβ segments, 6-7 Jβs and a single Cβ.

In mice 25 functional Vβ segments grouped into 20 families are also associated with 2 DJC clusters. These contain a single Dβ, 5 Jβs and a constant region [7].

7.2.2 What is the genomic organization of the locus?

In chapter 2, the *Xenopus* locus is depicted in cartoon form based upon which segments were associated with the *laevis* or *gilli* constant regions (see figure 2.7). Although we can infer which segments are found at each locus, our depiction does not imply that the arrangement of the segments in the locus are arranged as an array of Vβ segments upstream of two Dβ segments, followed by all of the J's and a single Cβ. This is likely if we compare *Xenopus* to the human and mouse TCR β loci, which are arranged with a cluster of Vβ segments upstream of two DJC clusters [7, 8]. However, unlike the axolotl, which possesses at least 4 Cβ segments, and mice and humans which have 2 Cβs, *Xenopus*, like chickens, possesses only a single Cβ region in its TCR β locus. How did *Xenopus* come to have only a single Cβ segment in its TCR β locus? *Xenopus* may have had a second Cβ segment, which was deleted, leaving it with a Dβ1–Jβ1, Dβ2–Jβ2 Cβ configuration. Alternatively, *Xenopus* may have had a single VDJC cluster, which became diversified as appears to be the case in trout, where genomic sequencing found a single

Dβ segment upstream of 10 Jβs and a single Cβ [9]. I would argue that the first option where a Cβ segment is lost is more likely. *Xenopus* has two Dβ segments that are extremely homologous to the two Dβ segments found in other studied vertebrates [4, 10]. Therefore, I predict that two DJ clusters will be found 5' of the single Cβ region. This would also account for preferential rearrangement of Dβ2 to Jβ3 (see chapter 5). The presence of large numbers of Dβ-J-C sterile transcripts in the absence of Dβ2-J-C transcripts also suggests that a promoter lies 5' of Dβ1 as is the case in mammals [5, 7, 8]. This also lends some credence to the deletion theory proposed above. A study of the genomic sequence of the *Xenopus* TCR β locus may help us understand the evolution of the locus.

7.2.3 The single well conserved Vβ segment

Three of the newly characterized *Xenopus* Vβ segments, 13, 14 and 19 shared significant (53-59% aa similarity) homology with a single well conserved Vβ segment, which is also found in humans (Vβ20), mouse/rat (Vβ14), shark (Vβ4), axolotl (Vβ5), and trout (Vβ1) [10]. It is intriguing to note that the mouse and human homologues are the only Vβ segments located 3' of the constant region and are transcribed in an orientation opposite to all other Vβs [8, 11]. Further studies in poikilothermic vertebrates could inquire if the same is true for the homologues present in these other species.

7.2.4 The H4 region appears to be under significant selection pressure

The variability of TCR Vβ segments was examined in chapter 2 as well as the substitution patterns between the two *Xenopus* species, which contributed to the LG hybrid. Non-synonymous substitutions were most frequent in the H4 region of Vβ segments. This region interacts with superantigens in mice and humans [12]. Superantigens (SAg) can lead to the non-specific activation of all T cells possessing TCRs, which use the same Vβ segment (see section 5.2.5). This strongly suggests that SAg continue to be an important selecting force in TCR Vβ regions of *Xenopus*. This may be a question of the number of T cells in the selected peripheral repertoire. Tadpoles possess a small, finite number of peripheral T cells. Superantigen activation of up to 20% of that small number of cells would likely represent a significant change in the animal's ability to protect itself from infection.

7.2.5 Poikilothermic vertebrates are likely to possess "ample" genomic repertoires of TCR β segments

Significant numbers of TCR β segments have only been cloned from the horned shark, the axolotl [10, 13, 14] and now exhaustively from *Xenopus* (see chapter 2 and [4]). If axolotl, shark and *Xenopus* with 13, 7, and 19 families of Vβ segments, respectively, are characteristic of the number of different Vβ families in other poikilothermic vertebrates, then it is clear that the number of TCR segments is of the same order as in mammals [15, 16]. The variability in these TCR Vβ chains was further tested by Shannon Entropy [17] analysis in chapter 2. Vβ segments in axolotl, shark, and *Xenopus* are as variable or more variable than Vβ segments in mice and humans.

More segments need to be cloned from poikilothermic vertebrates before we can truly compare the genomic repertoires of these animals. This is of course also a necessary step before the expressed TCR repertoires can be studied. As it stands, we can say that it is likely that most poikilothermic vertebrates will have an "ample" genomic repertoire, meaning that they will possess more genomic material for rearrangement than they can fully express in the peripheral repertoire of an individual (see section 7.7.4.2).

7.2.6 Further Work

TCR β segments fall into what has been termed by Klein [18] as an "immunological twilight zone". The twilight zone refers to homologies of 20-35% at the protein level. Often only the structural residues are conserved between two proteins but it is unclear if the proteins should be considered functional homologues. The future of comparative immunology requires that we do more than sequence comparisons and start to carry out structural or functional comparisons in order to establish "homology". As more and more genes are cloned from non-mammalian vertebrates through EST sequencing projects and as X-ray crystallography becomes faster, such studies become more plausible.

7.3 *Xenopus* T cells in ontogeny

7.3.1 Generation of TCR β diversity

The work presented in chapter 4 set out to add to our already substantial knowledge of T cell development in tadpoles. Previously the origin of thymocytes as well as the expression of several markers during T cell ontogeny had been studied. However, the onset of rearrangement was not known, nor was the extent of variability of segments used in early development and early tadpole life. During the course of this work, the onset of TCR β transcription and rearrangement was defined and the TCR β segments used during early development of tadpoles was determined. Staining with the polyclonal serum recognizing CD3ε, was used to determine the number and location of T cells in the young tadpoles (see chapter 4).

Next, we wanted to know how diverse the repertoire was in early life, and if programmed canonical rearrangements were found, like in chicken, or a wide diversity of unique T cells as was the case in mice and humans. [19-21].

Tadpole sections were stained with a polyclonal serum recognizing CD3ε to determine the number and location of T cells in the early tadpole. This allowed us to see CD3ε$^+$ T cells on day 5 in the lining of the stomach a full day before the first full TCR β rearrangements were found on day 6 in both thymus and liver/gut/stomach. The finding of TCR rearrangements and CD3ε$^+$ cells outside of the thymus at such early time-points strongly suggests extrathymic development of T cells in *Xenopus* tadpoles or TCR β rearrangements in immature T cell precursors homing to the thymus as has been described in mice and trout [22, 23].

The usage of Vβ segments in rearrangements isolated from tadpoles was quite random. None of our results suggests programmed developmental rearrangement at the *Xenopus* TCR β locus. As more and more cells accumulate in the tadpole, as shown by staining with the antiserum to CD3ε, more Vβs are detected by PCR, suggesting the repertoire of early tadpoles is limited by the number of T cells. All Vβs can be used in tadpole and adult life.

7.3.2 Are there NK cells in tadpoles?

Class I deficient mice possess natural killer (NK) cells. NK cells generally serve to kill cells which have down regulated class I MHC expression, but in class I deficient animals there appears to be a sort of NK education or mechanism of tolerance induction [24-28]. If such a mechanism exists in *Xenopus* tadpoles, NK cells may be present but anergic (unresponsive) to tadpole cells, which are class I negative or express very low numbers of surface MHC class I molecules. If this is the case, these cells are expected not to attack the B3B7 cell line which may have a low surface class I expression as suggested by RT PCR results presented in chapter 4. Protein expression of class I MHC protein was not proven but in light of the presence of class I mRNA, we cannot rule out low levels of class I protein expression at the cell surface. Thus previous studies which have concluded that NK cells are not present in tadpoles based upon experiments using B3B7 as a class I negative target must be considered inconclusive [2, 3]. The existence of NK cells in tadpoles remains a very plausible possibility. If NK cells exist in tadpoles they may provide an excellent comparative system for studying tolerance induction in NK cells.

This study of ontogeny also allowed an appropriate time-point to study the TCR repertoire of tadpoles. Tadpoles at 23-24 dpf were chosen for this study based on RT PCR results (shown in chapter 4) and previous studies of surface class I expression in tadpoles [29].

7.4 Generation of the TCR β repertoire

In chapter 5 we carried out the first full scale measurement of the TCR β repertoire in a poikilothermic vertebrate. As in mice and humans, both Jβ and Vβ usage is not fully random in the thymic repertoire.

Comparison of the thymic repertoires between tadpoles and adults showed significant differences in Vβ usage for both the LG3 and LG15 frog lines. Fewer differences were seen when the thymic repertoires of tadpoles were compared with tadpoles, or adults with adults. Therefore there seems to be a developmental bias in the usage of Vβ segments. This was attributed to either recombinational differences or a measurement of a mixture of selected and unselected T cells in the thymus. When an antibody to CD4 becomes available in *Xenopus*, it will be possible to sort populations of thymocytes more precisely, allowing us to differentiate these options.

The peripheral (splenic) repertoires differed between adult and tadpoles for LG3 but not for LG15, showing that MHC selection background can select distinct repertoires even when starting with the same TCR locus. These findings are difficult to interpret. However, mice and humans do not show significant differences between the fetal and adult repertoire [20, 30, 31]. The exposure of tadpoles to the outside antigenic world, unlike mice and humans that are protected in the uterus during gestation, may account for some of the differences in repertoire. Difference in class I MHC expression between tadpoles and adults may also account for the differences observed.

There were also significant differences seen in Vβ usage between LG3 and LG15 tadpole spleen repertoires and in between the adult spleen repertoires as well, confirming that the selecting MHC has a great influence on the peripheral repertoire.

7.4.1 T cell clone size in tadpoles

Based on the number of T cells observed in tadpoles using the CD3ε antiserum, and the diversity of sequences obtained from tadpole samples, the clone size (i.e. the number of cells that possess the same receptor because they are descendants of a single T cell) in tadpoles must be quite small. In this context, our repertoire results suggest that most T cells are unique. Immunoscope analysis will be needed to further determine the diversity of the repertoire by allowing the diversity of the CDR3 regions to be quickly determined on whole populations of T cells without requiring large scale sequencing.

7.4.2 Smart RACE will allow repertoire studies on very small numbers of cells

All future immunoscope analyses that are limited by small samples of T cells may benefit from the modified Smart RACE protocol presented here. When working with small numbers of T cells in the ontogeny study presented in chapter 4, it became clear that there was a problem with dilution of limiting samples. For instance, if one needs to characterize the diversity of a population of 50-100 cells, and one needs to run 19 Vβ specific PCR reactions in order to determine the diversity in the sample, significant diversity may be lost to the assay by dividing the template many times. This happens when looking at T and B cell ontogeny in *Xenopus*, but it is conceivably also a problem when characterizing other small T cell populations, such as intraepithelial lymphocytes or specific subsets of FACs sorted cells. I suggest that this efficient RACE protocol could be used to amplify the entire repertoire without introducing significant bias, so that sufficient template is available for spectrotyping.

As discussed at the end of chapter 5, this repertoire study was designed to provide a set of baseline repertoires, and to average individual differences. This leads to a lack of sensitivity. Therefore, if there are subtle but important effects to be found, we leave this to future studies.

This repertoire study is not as comprehensive or as conclusive as we had hoped. This is in large part due to the discovery of the 9 new Vβ and 12 new Jβ segments, which are reported in chapter 2. The discovery of the new segments nearly doubled the size of the repertoire study and greatly increased the number of T cells one would need to characterize to obtain statistically significant results.

7.5 Is class I MHC expressed during tadpole life?

It has been reported that, unlike adults, tadpoles do not express MHC class I of either the classical or non-classical types in the thymus [32-35]. Transcription of classical MHC I can be however be detected in the lungs, gills, gut and pharynx during tadpole life [29, 34-36]. In chapter 4, preliminary results are shown which suggest class Ia transcription can already be found at day 5 in LG15 tadpoles as well as in older day 15 and 23 tadpoles. This has implications for the repertoire study, because the tadpole repertoire was measured at day 23 or 24 postfertilization. It is possible that a small amount of class I protein could be produced from the transcripts we sequenced so tadpoles may not be completely class I negative after all. The PCR stategy used was not designed to be quantitative so the true amount of class Ia message at these stages is not known. Past studies have shown that at day 27 (stages 53-54) surface MHC class I cannot be detected on splenocytes, erythrocytes and thymocytes [29]. Low surface expression of class I molecules was detected in splenocytes, erythrocytes and thymocytes at day 34 (stage 55-56), day 46 (stage 57-58) and day 53 (stage 60), respectively [29]. $CD8^+$ cells are detected from 8 dpf onwards, well before surface expression of MHC class I molecules is detected in the animal. How then can we explain the selection of T cells with a $CD8^+$

phenotype in the periphery of tadpoles? Based on results in mice NK cell activity should also be affected by low amounts of surface MHC class I [28, 37].

The existence of $CD8^+$ T cells in the peripheral tissues of class I negative tadpoles, has been an enigma. Based upon the finding of class Ia transcription in very young tadpoles and mouse models that are "almost" class I negative, such as β_2m and TAP1 knockout mice, it is likely that the small number of $CD8^+$ T cells in *Xenopus* tadpoles are selected on very low levels of classical class I molecules that are undetectable by immunohistology.

In the past, immunofluorescence has been used to detect *Xenopus* class I molecules on the surface of cells [32]. Perhaps it is time to use a more sensitive technique employing antibodies labeled with colloidal gold and electron microscopy to see if there is a low density of class I molecules on the surface of tadpole cells.

If classical class I is not selecting $CD8^+$ T cells in tadpoles there is likely a non-classical class I molecule, which is responsible. In class Ia double knockout mice, a small population of $CD8^+$ T cells remained that were selected on β_2m-associated class Ib molecules. Crossing the double knockout mice to β_2m knockout mice completely blocked the selection of $CD8^+$ T cells [38]. It is still possible that a MHC linked nonclassical MHC class I molecule remains to be cloned which is the only histocompatibility molecule expressed at this early stage and which mediates the selection of $CD8^+$ T cells [39]. An alloantiserum against *Xenopus* tissue was reported which stained a cell surface molecule on the epithelium of the early thymus which was MHC linked but was not characterized further [32]. This molecule may be able to promote CD8 T cell selection.

The β_2m gene has recently been cloned from *Xenopus* (acc # BI477268). There are no reports about its expression during tadpole life. Several *Xenopus* EST projects are currently underway for tissues derived from tadpoles but β_2m is not present in among the ESTs which have been submitted to Genbank. This suggests that β_2m may also not be expressed during tadpole life and may be the factor which prevents surface expression of the transcribed heavy chain, much as is the case in β_2m knockout mice [40-42].

Next we asked what effect this dichotomy of class I expression had on the TCR β repertoire and why would a limitation of the MHC diversity be selected not only on an evolutionary time scale (genomic) but over the course of development?

7.5.1 Why do tadpoles and adults differ in their expression of class I? Some ideas...

If repertoire is not the major reason for modulating class I expression in development, what else could be driving such a difference to persist?

7.5.1.1 To prevent autoimmunity at metamorphosis

It has been hypothesized that because class I MHC presents intracellularly derived peptides, new antigens arising at metamorphosis might be recognized as foreign. It has been shown that tadpole T cells can make responses to adult cells [43]. In tadpoles primed to adult skin, haemorrhaging is detected in the skin of some newly metamorphosed animals presumably due to autoimmune responses. Still, at metamorphosis, there is a general immunosupression and naïve T cells undergo apoptosis [44]. One could argue that tadpoles could theoretically have a full class I and II restricted repertoire and eliminate this repertoire by directed cell death just before metamorphosis and not run the risk of autoimmunity. Because we see suppression at metamorphosis, in any case, the animal would not be putting itself at any further risk in such a scenario.

7.5.1.2 To increase the size and make-up of the helper T cell population

Perhaps class I expression is limited during tadpole life to increase the size of the helper T cell subset. In mammals, if either the CTL or helper T cell populations are compromised then the other compensates, in a process referred to as homeostasis (as reviewed in [45, 46]). By limiting the size of the CD8$^+$ population (compartment), tadpoles could be maximizing the number of helper T cells available in the animal [47]. This hypothesis is currently difficult to test, due to the lack of an anti-CD4 reagent in *Xenopus*, but seems reasonable in light of experiments where injecting adult T cells into tadpoles improved the efficiency of Ig switching implying that tadpoles lack T helper function [48]). However, it remains unclear if helper T cells in tadpoles and adults are qualitatively the same. We do know that adult T cells, unlike tadpole T cells, express class II MHC molecules on their surface, but the significance of this remains unknown. Although the introduced adult cells could promote B cells isotype switching of antibodies in tadpoles, this may only tell us that they are adult helper T cells rather than reflecting the function of the tadpole helper T cell subset. As new reagents become available, and new genes are cloned, it will be important to go back to this question again to determine if differences in helper T cell function in tadpoles are due to T cell numbers or qualitative differences in the nature of T cell help during development.

7.5.1.3 To provide the adult with predominantly class II restricted memory T cells

Perhaps the tadpole does not benefit directly from its T cell repertoire. It remains possible that tadpoles pass helper T cell memory on to the adult in order to provide a survival advantage to the adult. Improved immunity in adult frogs may improve reproductive success.

7.5.1.4 Do tadpoles rely on adaptive immunity?

Although there is considerable variability available to tadpoles their immune responses remain much less efficient than adult responses. This seems due to a limitation of the number of T cells in the periphery that can carry this variability.

In light of this limitation, it is questionable whether the tadpole actually relies on its adaptive immune responses for protection. The animal is so small and its repertoire so limited that, if attacked by viruses or bacteria, it is difficult to understand how the tadpole could afford to wait for an adaptive immune response. The pathogen, which can multiply much more quickly, should overwhelm the tadpole at that point. We must of course remember that we still do not understand the role of $\gamma\delta$ T cells during tadpole life, and these cells may play a very significant role in protection from pathogens. Innate responses must play an important role in tadpole immunity. If this is true then why spend the energy to develop a "special" case of adaptive immunity without the benefit of MHC class I during tadpole life?

Perhaps this is a question of memory. A small population of tadpole memory T cells persist in the adult when the rest of the tadpole naïve T cell population dies via corticosteroid induced apoptosis.

Maybe the only reason a tadpole adaptive immune response exists is, as discussed earlier, to pass along a predominantly helper T memory to the adult [49]. It might also be possible that class II restricted T cells selected in the class I deficient tadpole may be passed as memory cells to the adult. A T cell with the same receptor in the adult might be selected into the class I restricted CTL subset. There might be an advantage to have both class I and II restricted T cells with the similar specificities and might actually expand the usable peripheral repertoire somewhat in young adults.

7.5.1.5 To produce an atypical population of T cells with a specific function

In mice, when MHC class I is reduced or knocked out, there are certain T cells which enter the periphery as CD4s which would differentiate into the $CD8^+$ subset in the presence of class I [50, 51]. Perhaps in *Xenopus* tadpoles the same is true allowing tadpoles to select a set of helper T cells that would normally be part of the CTL subset.

However, the results presented in chapter 5 suggest that the developmental modulation of class I MHC expression is not to select for usage of a specific repertoire of segments in tadpole or adult life, or at least not to the extent that our repertoire study would detect. It remains unclear why class I MHC expression changes during *Xenopus* development and further work will have to address this. A good starting point would be to introduce a class I MHC transgene into tadpoles and see what effect this has on NK and T cells.

7.6 Selection pressures on the number of TCR β and class I MHC loci

7.6.1 Are all Class I genes equivalent?

The number of class I genes in most vertebrates seems quite low, on the order of 2 or 3 loci. However, quite a few organisms (chickens, *Xenopus*, dogs, rabbits) seem to predominantly express only one MHC class Ia locus [49, 52, 53]. Where there are multiple expressed class I genes do all of them affect the selection of the T cell repertoire to the same extent? Recent results suggest that the K^b and D^b genes in mice are not equivalent on their effect on deletion of T cells. K^b and D^b knockout mice respectively have 30 and 60% reductions in the number of $CD8^+$ T cells in the periphery as compared to wild-type mice. Double knockout mice have a 90% reduction [37]. This finding must change the way we view MHC loci in other animals as well. If the MHC molecules in an individual are not functionally equivalent, we will not be able to directly correlate the number of MHC molecules with their effects on T cell diversity in the periphery.

Is there an evolutionary reason for this seeming reduction in potential diversity?

7.6.2 Is there a limitation on the number of functional TCR β or MHC Loci?

Speciation in *Xenopus* has often involved the production of polyploids (via allopolyploidization). These polyploid individuals are hybrids of different species of *Xenopus*. Thus, in the wild we find 2N=20 chromosomes (e.g. *tropicalis*), 4N=36 chromosomes (e.g. *laevis, gilli, muellerii*, 8N=72 chromosomes (e.g. *vestitus, wittei*), and 12N=108 chromosomes (e.g. *ruwenzoriensis*) species ([54] and as reviewed in [55]).

The number of active loci has been defined for many genes in these species, allowing us to observe the effects of millions of years of selection on genes of the immune system. Except in the most recent polyploid species with 108 chromosomes, the number of MHC class Ia loci in *Xenopus* is 1 per haplotype whatever the level of ploidity. Clearly, the MHC class Ia genes show a trend towards diploidization. Similarly, in all species, except again the relatively recent polyploid, *X. ruwenzoriensis*, the number of TCR loci has also appears to have been reduced to one locus per haploid genome, suggesting there may be a functional significance to this evolutionary reduction to diploid inheritance of both class I MHC and TCR β loci ([4] as reviewed in [56, 57]).

7.6.3 Why do we see this reduction in MHC and TCR loci?

A reduction in the number of MHC and TCR loci could be due to limitations on the density of specific MHC molecules on the cell surface and problems associated with allelic exclusion.

7.6.3.1 A problem with density of each gene product on the cell surface?

MHC genes are co-expressed. Thus, an increase in the number of MHC alleles expressed in an individual decreases the relative surface expression of each allele on the cell surface. Less MHC on the surface means that individual T cells will have a more limited number of MHC molecules to interact with which may lead to problems in activating T cells.

Others have argued that expression of many MHC alleles would lead to the negative selection of more T cells in the thymus which would lead to less T cells in the peripheral repertoire and limit the diversity of TCRs present in the periphery. This would be especially problematic for animals with a small T cell repertoire because it would decrease the size of an already small peripheral repertoire.

7.6.3.2 Problems with allelic exclusion?

Generally only one functional TCR β gene is produced per T cell, through the control of a process called allelic exclusion. However, in polyploids, what happens when there are suddenly 4 or 8 loci?

It has also been argued that the TCR may be diploidized due to some problems associated with allelic exclusion of "extra" loci [60]. The mere presence of extra TCR loci is unlikely to overwhelm the allelic exclusion machinery because allelic exclusion still works at the Ig loci that have not been diploidized [60].

Allelic exclusion at the TCR locus has not been studied in *Xenopus* species. It is likely that excess TCRβ loci would not lead to problems but a lack of efficient allelic exclusion at the TCRα locus could lead to T cells with multiple surface receptors. In humans and mice, cells with two receptors exist in normal individuals but are less likely to be activated in the periphery because they are less sensitive to antigenic stimuli due to a reduced surface density [61, 62]. This lower sensitivity could actually lead to the selection of TCRs, which, if expressed alone, would lead to the negative selection of the cell [63]. Could this lead to a reduction in the number of α loci? The TCR α chain has recently been cloned in *Xenopus*, but there is no information available yet concerning the number of TCR α loci present (Haire et al, accession # AF440803-AF440817). It seems likely that this locus, like the TCR β locus, will be diploidized.

Future repertoire studies need to address what happens to T cell selection in lab produced polyploid frogs. Single cell PCR will be needed to determine if more than one receptor is being produced per T cell. If so, the repertoire of such a T cell population should be tested for diversity. This could help to clear up the contraversy as to if the expression multiple TCRs increases [64] or decreases [62] the effective peripheral repertoire. A possible complicating factor to this is that polyploid frogs tend to be smaller than diploid or pseudotetraploid species and yet their cells are bigger. Fewer cells coupled with expression from more loci could lead to problems.

7.7 General questions of diversity in the TCR repertoire

As outlined in the introduction, we must study diversity in the TCR β from several viewpoints. Each vertebrate starts off with a set number of genomic segments. We have termed this the genomic repertoire. The degree of diversification, which is introduced during somatic DNA recombination, gives us the pre-selected repertoire. Third is the theoretical peripheral repertoire, which is composed of T cells with TCRs, which can be selected on a particular MHC background. Fourth is the peripheral selected repertoire, which reflects how much of the selected repertoire can be expressed in the peripheral repertoire.

7.7.1 The peripheral repertoire: does size matter?

As stated earlier, the genomic repertoire of TCR β segments in axolotl, *Xenopus* and shark is quite ample when formally compared to mice and humans using Shannon Entropy analysis (see chapter 2). Preliminary results in (trout and catfish) suggest that other poikilothermic vertebrates will be similar but more TCR segments from these species are required before we can draw any conclusions [9, 65-69]. If the genomic repertoire of TCR β segments were also as extensive as in mice and human, *Xenopus* would be capable of expressing much the same diversity as in mice and humans. However, even adult frogs tend to have fewer lymphocytes than mice, therefore cell numbers may still limit the peripheral selected repertoire.

7.7.2 How many segments does an individual require to produce an adequate repertoire?

Casrouge et al, [70] have calculated that the potential diversity of TCRs in mice is 10^{15} but the individual mouse only can rearrange 10^{7} TCRs in the cells it possesses. If only $2x10^{6}$ selected T cells leave the thymus each day that it would take $\sim 10^{9}$ days to use the entire "potential" genomic repertoire [70].

Clearly even if the true rearranged pre-selected repertoire is many times smaller than the theoretical 10^{15} due to non-random usage of TCR segments or inefficiencies in the pairing of α and β chains, most individuals will still possess much more "raw material" in their genomic repertoires than they will actually use. This is why I suggest that poikilothermic vertebrates are likely to have "ample" genomic repertoires.

7.7.3 How much diversity is needed in the expressed peripheral repertoire?

The theoretical peripheral repertoire is composed of all TCRs, which can be selected on a particular MHC background in an individual (keeping in mind that a slightly different population of peptides will be presented by MHC in each individual). The theoretical repertoire is a misleading number because it does not take into account that, at any particular time, there are a finite number of cells in the periphery, which can carry those receptors.

The size of the peripheral repertoire is important because it limits the variability, which can be expressed in an individual at one particular time. Thus, small animals with limited numbers of T cells are expected to carry a less diverse, but also potentially more dangerous (because less diversity implies more cross reactivity) collection of receptors than larger animals. It also implies that there might be a selection in larger animals for greater genomic diversity, because these animals may possess enough cells to carry this "additional" diversity. The number of cells carrying a particular receptor is also a factor when considering the diversity of the peripheral repertoire.

7.7.4 The elephant vs. tadpole paradox

This balance is referred to by Langman & Cohn (1987)[71,72], as the E-T or elephant versus tadpole paradox. They argue that there must exist a common ratio between the size of an animal and the variability and number of cells an animal must possess to have a similar minimum level of immune function. For example, elephants and whales have more T cells than tadpoles, hummingbirds or mice do, but also have a larger area for those cells to monitor. Langman & Cohn have dubbed a level of immunity that will protect the organism in over 90% of cases a protecton [72]. The smallest vertebrates with enough immune variability and immune cells to reach this level of immunity are said to possess a single protecton. Through an esoteric set of calculations based only on B cell immunity and on very soft numbers, Langman & Cohn claim that a protecton is roughly equal to 1 X10^7 B cells. Hummingbirds and mice would therefore possess a single protecton whereas an elephant would then need 10^7 protectons for an equal level of immunity. Tadpoles are deemed to have only 10^{-1} protectons and are expected to show considerable mortality during larval life therefore necessitating the production of huge numbers of individuals to allow passage of a few individuals through this "immunological bottleneck" (Langman & Cohn, 1987 p.679)[71].

The protecton model, though deeply and irreconcilably flawed (see biting critiques in [72-75]), was a valiant attempt to begin a discussion about how much variability an organism needs for adequate protection and suggested that evolutionary selection could compensate to some extent to the individual needs of each species.

7.7.4.1 The tadpole component of the E-T paradox

This thesis presents the first data that approach the tadpole component of the E-T paradox from a T cell point of view. With their limited size and number of T cells, *Xenopus* tadpoles arguably possess one of the smallest (many fish are also very small and would be expected to possess very small repertoires as well) T cell repertoires amongst vertebrates. This work has shown that tadpoles can use all Vβ segments and suggest that the tadpole peripheral repertoire is as variable as that of adults in that tadpoles can express all TCR β segments. This study cannot provide a complete estimate of variability in *Xenopus* for there is no data available concerning the diversity of the TCR α chain, and due to technical constraints we did not address the repertoires of single tadpoles. It is also quite difficult to address the E-T paradox without information concerning the elephant. However, while the TCR remains Uncharacterized in elephants and whales, good data is available for mice and humans.

7.7.4.2 Measurements of peripheral repertoire in mice and humans

In mice and humans, estimates of the actual αβ TCR repertoire have been made. Casrouge et al (2000)[70] have estimated the actual repertoire size in mice to be 1-2 X10^6 TCRs in 2x 10^7 cells. Based upon a similar study Arstila et al,[76] have estimated the human repertoire to be repertoire to be 2.5 X10^7 different receptors. Casrouge et al [70], have noted that this corresponds to a difference in diversity of only 10-20 times "even though there are 250 times more T lymphocytes in human blood than in a mouse spleen" ([70],p. 5787). There is therefore no proportional increase in diversity seen with an increase in size, and Casrouge et al invoke the concept of the "protecton" unit put forth by Langman and Cohn [70][71]. Perhaps there is a minimum functional number of T cell specificities needed in the individual, which is repeated in larger organisms. If there is a minimum "protection" unit, is this unit based upon the smallest vertebrates such as fish and tadpoles which are free living from early in development or is the protecton, as Langman and Cohn propose, specialized for vertebrates larger than mice? They have proposed that tadpoles because they lack "a protecton" may need to produce large numbers of individuals in order to compensate for the death of a part of the tadpole population due to infection.

7.7.4.3 Further Work

With the recent cloning of the *Xenopus* TCR α chain (Haire, R.N et al, direct Genbank submission, 2001) it is now possible to test the "E-T paradox" by measuring the actual TCR $\alpha\beta$ repertoires of tadpoles and of adult *Xenopus*). Because the analysis is dependent on immunoscope PCR, this could prove problematic with small amounts of starting materials. I propose that the 5' Smart RACE technique used in chapter 5 would allow the repertoire to be preamplified without introducing significant bias. Immunoscope analysis could then be performed on small samples.

7.7.5 The size of the expressed TCR repertoire is also dependent on MHC expression

Because it is the peripheral T cell repertoire under selection pressure, not only is the genomic variability at the TCR under selection pressure but also the variability and number of MHC loci expressed in the organism.

Xenopus polyploid species provide a unique opportunity to study several natural variations in MHC expression and long term (i.e. millions of years) selection pressures on the MHC and TCR loci thus offering some titillating clues to how selection balances these factors.

7.7.6 Do smaller animals have more cross-reactive T cells?

It has been suggested that the immune system must be sufficiently cross reactive to allow it to maximize the number of foreign antigens that it can recognize yet specific enough that it will be able to differentiate self from non-self and be able to respond to the antigens with a memory of an appropriate secondary immune response. Appropriate here means that the lymphocyte not only stores specificity but also stores the memory appropriate type of immune response for that situation.

This sort of model has implications for immunity in *Xenopus* and in poikilothermic animals in general.

We expect that if large and small animals possess T cells with the same level of specificity and cross reactivity, then smaller animals with less lymphocytes would be less likely to recognize an antigen than larger animals. This limitation could be partially overcome if smaller animals had more cross reactive T cells than larger animals allowing them to maximize the reactivity of those cells. This would also imply that small animals would not be able to make as precise a distinction between self and non-self and predicts autoimmunity to be problem in smaller vertebrates [77, 78].

7.7.7 The effect of temperature on the specificity and maintenance of T cell memory

In chapter 6, theoretical ideas concerning T cell specificity and temperature are presented. To my knowledge this is the first time that this issue has been raised for poikilothermic organisms. Some of the principal ideas of our current understanding of T cell selection and specificity are challenged when viewed from the point of view of a poikilothermic creature that undergoes ambient temperature fluctuations. Here I have suggested that temperatures shifts of only 5-10°C could change the fine specificity of TCRs, and that this implies that a portion of the usable selected peripheral repertoire is lost to non-responsiveness or anergy with both increases and decreases in temperature. It is also implied that the TCR can undergo changes in specificity that are, in a way, similar to somatic hypermutation of antibodies (immunoglobulins).

Studying the loss of a portion of the repertoire to anergy or non-responsiveness will be challenging. If cells remain alive but non-functional it will be difficult to measure their repertoire and differentiate it from active T cells.

7.8 Final Words

The vertebrate adaptive immune system is an example of evolutionary variation on a conserved theme. Although all vertebrates possess the same basic units of adaptive immunity, each species has adapted these in slightly varying ways. This provides us with an incredible opportunity to observe the outcomes of evolutionary experiments that have been underway for millions of years. Comparisons of adaptive immunity in different vertebrates will help to define the essential core elements of the vertebrate adaptive system as well as the unique adaptations in each species.

This thesis addresses the issue of diversity in the adaptive immune system by providing the first comprehensive look at diversity in the TCR β chain in a poikilothermic vertebrate. The larger question of how much diversity a creature needs in its BCR and TCR repertoire to mount effective adaptive immune responses remains unanswered. To address this question from the perspective of the TCR, the genomic repertoires of TCR β segments need to be cloned from more vertebrates and the usage of these segments in relation to the number of cells present in each organism needs to be studied.

Finally, I have raised the question of the effect of temperature on the peripheral TCR repertoires of poikilotherms and heterotherms. This theory predicts that both increases and decreases of temperature should decrease the usable TCR peripheral selected repertoire. Experiments to test this, although technically challenging, need to be carried out to determine the actual size of the peripheral TCR repertoire in vertebrates that undergo changes in body temperature.

The best model in which to test this theory of temperature effects on T cells is the channel catfish. Temperature effects are already well defined in the catfish and T cell lines are available allowing *in vitro* studies of T cell function to be carried out.

The *Xenopus* system can be used to advantage to study diversity in the immune system as well as the selection of T cells. *Xenopus* development and immunology is well defined. The availability of isogenic lines of frogs and immortalized tumour cell lines makes the *Xenopus* model attractive. Perhaps most importantly, transgenes can now be introduced into *Xenopus* allowing the effects of gene dosage to be studied and compared [79]. The baseline TCR β repertoires presented in chapter 5 will allow the *Xenopus* system to be used for studies of how gene dosage affects repertoire. Trangenesis can now be used to knock in rearranged TCR α and β chains to produce animals with only a single TCR specificity or extra copies of MHC genes. We can now study the effects of multiple TCR and MHC loci present in lab produced polyploids on the selection of the TCR β repertoire. This production of polyploid hybrid frogs closely mirrors a natural process of hybridization in the wild, which leads to the formation of new *Xenopus* species. A study of repertoire in these animals will give us insights into the selection pressures on the T cell repertoire of new *Xenopus* species.

Many of the ideas put forth in this thesis are just that- ideas. The temperature model is a theoretical model based upon circumstantial evidence at best, but it provides a context in which to move forward in our understanding of the evolution of T cell function. In the words of Jonas Almeida, "All models are wrong but some models are useful" [80]. A useful model is testable and provokes discussion and experimentation. My primary purpose in putting forth this model and presenting this work concerning TCR diversity in *Xenopus* is to reopen the discussion on the effect of temperature on T cells and on the larger issue of diversity in the immune system, because many new experimental techniques are now available to look at these questions from a slightly different perspective than we could 10 or 20 years ago. Many of the ideas and predictions put forth in this thesis may, in a final analysis be proven wrong, but I hope that the model will provoke the work which will prove them right or wrong or suggest completely different options.

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Appendix A

Materials and Methods

Appendix A. Materials and Methods

A1 Metabolic ^{35}S labeling of the B3B7 cell line

1). Collect log phase B3B7 cells were collected and pellet (1000 rpm 12min), rinse 2x with RT or 27°C amphibian PBS (APBS), count and score for a minimum 80% viability.
2). Resuspend cells at ~2.5 x 10^{7} cells/mL in pre-warmed (27°C) labeling media without ^{35}S Cys/Met
3). Add 200 µl of ^{35}S Cys/Met (Amersham Redivue PRO-MIX : L- {^{35}S} *in vitro* cell labeling mix 7.15mCi/ 500µl) per 2 mL labeling media.
4). Label cells 2 hours at 27°C with gentle agitation every 0.5 hours.
5). Centrifuge at 1000 rpm at 4°C and remove supernatant
6). Rinse cells 5x with 12 mL of 4°C APBS.

Labeling media

Cell culture Water (Sigma)	785 µl
L-Glutamine	65 µl
dialyzed FCS	650 µl
Cys/Met/L-Glut free Dulbecco's Modified Eagle's Medium (Sigma)	5 mL

Digitonin Lysis of B3B7 cells

1). Lyse cells in 1x lysis buffer at 1X 10^{8} cells/mL
2). incubate on ice 1 hour
3). centrifuge at maximum rpm 15 min
4). remove supernatant and discard the pellet

Lysis buffer for immunoprecipitation (use at 1X 10^{8}cells/mL)

1% digitonin (2% stock)	25 mL
2x lysis buffer	25 mL
Complete™ protease inhibitor (Roche Diagnostics)	2 tablets
	ad 50 mL

2x Lysis Buffer

Stock	Final	200mL	50mL
5M NaCl	0.3M	12mL	3mL
0.5M Tris pH 7.4	0.1M	40mL	10mL

0.5M EDTA	0.01M	4mL	1mL
10% NaN_3	0.2%	4mL	1mL
Iodoacetamide	0.04M	8mL	2mL
		ad 200	ad 50mL

Iodoacetamide 1M stock: 1.85g/10mL (make fresh each month; when it turns yellow it has gone bad,TOXIC!)
2% digitonin: percentage calculated by weight; takes several days to go into solution, extremely TOXIC!!!

A2 Immunoprecipitation

A2.1 Immunoprecipitation using antibody coupled sepharose beads

Coupling of Antibodies to CNBr - activated Sepharose Beads (CNBr activated Sepharose beads 4B Pharmacia)

1). weigh powder and suspend in 1mM HCl (1g freeze dried gel yields 3.5 mL gel)
2). wash using approx. 200 mL 1mM HCl / 1gram powder
3). ligand must be in coupling buffer (no TRIS!)
    -coupling buffer= 0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl
    -dialysis tubing with a cutoff of 15000 kD for at least 2 hours at 4°C
4). take the OD_{280} of the antibody solution (for Ig couple a maximum of 5 mg/mL) before coupling the beads
5). mix the coupling solution with the beads and rotate 4°C ON
6). spin the beads down (1000-1500 rpm) and take the OD_{280} of the sup
    $\frac{1OD280}{1.4}$ = mg/mL for antibodies
7). wash several times with coupling buffer (a minimum of 5X the volume of the gel)
8). wash alternating A and B
    A. 0.1M acetate buffer pH 4.0 with 0.5M NaCl
    B. 0.1M Tris buffer pH 8.0 with 0.5M NaCl

Immunoprecipitation

1). incubate lysate with 10 µl antibody coupled to sepharose beads (see above for coupling procedures)
2). ON rotation at 4°C
3). spin beads down, remove lysate
4). rinse 10x with lysis buffer
5). rinse 2x with PBS
6). add SDS sample buffer (need 20-25 µl sample) and incubate at RT for 20 min
7) centrifuge and remove supernatant which should contain the protein of interest
7). boil samples 5 min then load onto 4-12% NuPAGE precast gel run with MOPS running buffer
    -voltage = 200V constant
    -current = 100-115 mA/ gel
    - = 60-70 mA/ gel
    - run time ~ 50 min

A2.2 Immunoprecipitation on a solid substrate (ELISA plate)

Coating the plastic (ELISA) plates with the antibody

1). place 100 µl of the secondary antibody dilution in each well of the ELISA plate and incubate ON at 4 °C

2). wash 3x with 0.05% Tween in PBS
3). add 80-100 μl of dilution of the primary antibody to the well and incubate ON at 4 °C
4). wash 3x with 0.05% Tween in PBS
5). block 1 hour at 37°C with 1.5% BSA in PBS
6). add lysate to the well
7). incubate 2hours at 37°C
8). wash 3x with 0.05% Tween in PBS
9). elute the sample from the plate surface by

solution A (20mL is enough for one plate)

8.42 mL	0.2M	Na_2HPO_4 x $2H_2O$	(17.6g/ 500mL)
9.58 mL	0.1M	Citric acid	
2.0 mL		ABTS	(0.05g/ 5mL H2O)
100 μl	1%	H_2O_2	

Borate saline

12.37g	H_3BO_3	Boric acid
19.08g	$Na_2B_4O_7$ X 10 H_2O	sodium borate
8.77g		NaCl
ad 2L		

pH should be ~8.0-8.2 without adjustment
(a 5X solution is possible but needs to be heated)

Immunoprecipitation

1). place 100 μl of lysate into the well of an ELISA plate with the antibody coupled to the surface
2). incubate ON at 4°C
3). remove lysate from well
4). rinse the well 10x with lysis buffer
5). rinse the well 2x with PBS
6). add SDS sample buffer and incubate at RT for 20 min
7). remove supernatant that should contain the protein of interest

A3 Histology

Tadpoles were fixed for 1 week in freshly prepared 4% paraformaldehyde, embedded in plastic resin according to the manufacturers instructions and cut into 6μ sections. Sections were stained with Modified Nocht's Azure-eosin stain.

Modified Nocht's Azure-eosin stain

8.0 mL	0.1% azure A,
8.0 mL	0.1% eosin B
0.1M	citric acid
1.2 mL	H_2O

ad 25mL dH_2O. pH to 5.2 with disodium phosphate and add 5.0 mL of acetone

A4 Immunofluorescence

1). Whole tadpoles, adult tissues and tumors were frozen in O.C.T. embedding medium (Tissue Tek), cryosectioned (6μ), fixed 1 minute in acetone
2). block slides with 1.5% BSA in PBS for 1 hour
3). A 1:25 dilution of the rabbit anti-human CD3ε antiserum (DAKO A0452) was absorbed twice volume to volume on *Xenopus* red blood cells. The absorbed serum was further diluted 1 to 100 and incubated with the sections for 2 hours at room temperature.
4). wash sections 3 times in PBS and again block with BSA

5). Incubate with FITC or TR labeled goat-anti-rabbit Ig (SBA, Birmingham, AL) antibody (1:200 dilution) for 30-60 min.

6). Anti-CD8 staining was performed with the AM-22 mAb (1:75 dilution) followed by a fluorochrome conjugate of goat-anti-mouse-IgM (SBA).

A5 RNA isolation

1). TRIZOL (GibcobRL) was used for RNA isolation according to the manufacturer's instructions except that 15µl of water per 1ml Trizol was added to the initial homogenization step and that glycogen was used as a carrier in all precipitations.

A6 cDNA production

1). Mix the following:

x µl	total RNA (0.5-0.7µg)
2 µl	hexamers (500 µg/mL stock)
ad 12 µl	

Note: all reagents must be RNase free.

2). heat 3 min at 70 °C in heated lid thermocycler
3). spin briefly and incubate on ice for 1 min
4). add 8 µl of reaction mix to each rxn

Reaction Mix:

2.0 µl	dNTPs (10 mM of each dNTP; Na+; NEVER Li^+)
2.0 µl	10x RT buffer
1.0 µl	RNasine (Promega; 40 000 units/mL)
2.0 µl	DTT (0.1M)
1.0µl	Reverse transcriptase (RT)(Promega or Gibco-BRL: MLV-R)
8.0 µl	

5). 45 min at 37 °C in heated lid thermocycler
6). spin briefly
7). 4 min at 92 °C (inactivates RT)
8). spin briefly
9). dilute in 0.5X TE
10). aliquot and store at –20—70 °C.

Note: the appropriate control for further PCR on such cDNA preparations is to prepare a replicate cDNA synthesis reaction without the RT. You can test for contamination by genomic DNA or plasmid specific primers.

Reverse Transcriptase 10X Reaction Buffer:

0.5 M	Tris-HCl (pH 8.3)
0.1 M	$MgCl_2$
0.75M	KCl

A7 Basic PCR protocol

2). **Mix 1:**

CB primer (AS) (17707)	2.0 µl
V beta specific sense primer	2.0 µl
dNTPs (200 µM) Li^+	5.0 µl
Elmer 10x Buffer	5.0 µl
*Ampli*Taq DNA polymerase	0.7 µl
H_2O	35.3 µl
	49.0 µl

PCR thermocycling is as follows:

25-30 cycles of :

94°C	5 seconds
59°C	10 seconds
72°C	2 min

Followed by 70°C for 10 min
Hold at 4°C

A8 Multiplex PCR

(see figure 4.7 on page 182.)

A first round of PCR employs multiple Vβ primers (1µl each) and a single constant region primer (5µl). All other PCR condition are the same as in the PCR protocol given. After 20-25 rounds of amplification the reaction is split into separate reaction tubes, each containing a nested primer for a single V and a constant region primer and submitted to a PCR reaction with the same basic protocol as above. Vβ presence or absence assayed on an agarose gel or by sequencing. Each set of primers need to be optimized. Great care was taken to keep PCR conditions uniform and to prevent contamination. Master mixes for each of the Vβs were prepared and split into positive (internal Cβ and adult thymus) and negative controls (water controls for both the first and second rounds of PCR and egg or cDNA from day 2 whole embryos) as well as for the conditions tested. A subset of the products were blotted and filters hybridized by Cβ probe or Vβ specific probes to ensure the fidelity of the primers under these amplification conditions.

A9 Smart RACE library construction

Kit: Clontech SMART RACE cDNA amplification kit (K1811-1) (see diagram on page XXX)

1). RNA was isolated using TRIZOL (see protocol included above) and stored at -70 °C

2).

500 ng of total RNA	Xµl
5 CDS primer	1µl
Random hexamer	1µl
	ad 4µl

3). Heat this mixture at 70 °C for 2 min in a thermocycler with a heated lid

4). Centrifuge briefly in a cold centrifuge
5). Incubate on ice for 2 min
6). Add 5µl of Mix A

Mix A:	2µl	5x 1st strand buffer
	1µl	DTT (20mM)
	1µl	dNTP Mix 10µM (must be Na^+ based)
	1µl	RNAse H^- reverse transcriptase (Superscript II) (200U/µl)
	5µl	

7). Incubate for 2 min at 42 °C in a thermocycler with a heated lid
8). Add SMART II oligonucleotide or other primer with a 3' poly G tract
9). Incubate for 1.5 hours at 42 °C in a thermocycler with a heated lid
10). Heat to 72 °C for 12 min to inactivate the reverse transcriptase
11). Dilute RACE library appropriately.

Reagents

Random hexamer: 0.117 µM/ml
5' CDS (anchored poly T primer): 5' –$(T)_{25}$ N_{-1} N-3 where N = A, C, G or T and N-1 = A, G or C.
SMART II oligonucleotide: 5' AAGCAGTGGTAACAACGCAGAGTACGCGGG –3' (10µM)
DTT : 20 mM
dNTP mix : 10 mM each of: dATP, dGTP, dCTP and dTTP

(do not use nucleotides in Li^+ buffer as the Li^+ inhibits the reverse transcriptase)

5x 1st strand buffer (NOTE:This is not the buffer provided with the SuperScript II reverse transcriptase)

250 mM	Tris – HCl (pH 8.3)
375 mM	KCl
30 mM	$MgCl_2$ (~5x the [$MgCl_2$ of the buffer supplied with the enzyme)

A10 RACE PCR (Touchdown PCR)

1). 1µl diluted library is mixed with 49 µl of mix. (everything must be on ice)
2). **Mix 1:**

CB primer (AS) (17707)	2.0 µl
(UPM) universal primer mix (10x)	5.0 µl
dNTPs (200 µM) Li^+	5.0 µl
Elmer 10x Buffer	5.0 µl
Taq DNA polymerase	0.7 µl
H_2O	32.3 µl
	49.0 µl

Reagents: CB primer (17707): 5'-ATTCTTGGTGAGGCTGAGGCGGCTGC-3'
UPM: CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT–3' (0.2µM)
and CTAATACGACTCACTATAGGGC (1µM)

GeneAmp 10x PCR Buffer (Elmer Buffer)
(Perkin-Elmer)

KCl	500 mM
Tris-HCl pH 8.3 (at 25 C)	100 mM
$MgCl_2$	15 mM
gelatin	0.01% (w/v)

*Ampli*Taq DNA polymerase 5 units/µl and

3) Mock hot start PCR (e.g. reactions are on ice and thermocycler is heated to 94°C and reaction tubes go directly from 4°C to 94°C.

5 cycles of:

94°C	5 seconds
68°C	10 seconds
72°C	2 min

Followed by 5 cycles of:

94°C	5 seconds
65°C	10 seconds
72°C	2 min

Followed by 25 cycles of:

94°C	5 seconds
63°C	10 seconds
72°C	2 min

Followed by 70°C for 10 min
Hold at 4°C

4) Take an aliquot of 1 – 2 μl from this reaction and dilute it 1:10, 1:50 and 1:100. Use 1μl of these dilutions for the second round of PCR

1μl dilution + 49μl of Mix 2

Mix 2:

CB primer (AS) 17707	2.0 μl
NUP nested universal primer	5.0 μl
dNTPs (200 μM)Li^+	5.0 μl
Elmer 10x Buffer	5.0 μl
*Ampli*Taq DNA polymerase	0.7 μl
H_2O	32.3 μl
	49.0 μl

NUP (nested universal primer) (10μM): 5'- AAGCAGTGGTAACAACGCAGAG T-3'

5) Use a mock hot start or a hot start for the second amplification

The cycling conditions are as follows:

25 to 30 cycles of:

94°C	5 seconds
63°C	10 seconds
72°C	1 minute

Followed by 10 min at 70°C

6). PCR products were separated on a 1% agarose gel.

PCR products were then separated on a 1% agarose gel and bands larger than 450 bp were purified using Geneclean (BIO 101) kit which employs glass bead purification of DNA in a spin column format.

A11 Subcloning

Samples were eluted in water and ligated into a T-tailed PCR II TA vector (Invitrogen) using a traditional ligation as per the manufacturer's protocol. (NOTE: TOPO cloning is not appropriate here for it favors the cloning of small PCR products). Ligations were transfected into chemically competent TOP 10 bacteria, plated and grown overnight on LB plates containing ampicillin.

A12 Plasmid Minipreps

Commercially available spin columns (Wizard Minipreps (Promega) and QIAGEN) based on the alkaline lysis protocol for plasmid minipreps were used to purify plasmid for sequencing.

A13 Sequencing

Sequencing of double stranded DNA (dsDNA) was carried out on an ABI sequencer using the Big Dye Terminator Kit and on a LI-COR system using fluorescently (MWG Biotech) labeled primers and a thermosequenase kit using standard protocols. Standard primers T7, M13 forward, M13 reverse and T3 were used for sequencing. Sequences were then blasted against a local sequence database using software developed by Stefan Meyer. Sequence alignments were generated using either the Lasergene multi-sequence alignment tool or CLUSTAL using a PAM 250 matrix.

A14 Southern Blotting

A14. 1 Labeling Probe

Prime-It II Random Primer Labeling Kit (Boehringer Mannheim)

1). Mix the following
    25 ng DNA template
    X µL H_2O
  ad 23 µL
    10 µL random oligonucleotide primers (9mers) 27 OD units/mL
2). heat at 95 °C for 5 min and cool on ice
3) Add to this mixture:
    10 µL 5x primer buffer (without dCTP, 0.1 mM of dATP, dGTP, dTTP).
    5 µL labeled α ^{32}P dCTP (Amersham ^{32}P α dCTP (AA0005))
    1 µL Exo (—) Klenow enzyme (5U/µL).
4). incubate at 37 °C 2-10 min.
5). add 2 µl of stop mix (EDTA 0.5 M, pH 8.0)
6). purify away from unincorporated dCTP using a push or spin column
    (e.g. Stratagene NucTrap Probe purification column (400701)).

A14.2 Southern Blotting

1). Separate DNA (10µg of genomic DNA digested to completion with restriction enzyme of choice) on a 0.8-1.0% agarose gel without EtBr in fresh 1x TAE overnight (12 hours) at 50V.
2). Treat gel for 20 min with 0.5 M NaOH; 1.5 M NaCl
3) Rinse gel with water

4). Treat gel with 0.5 M Tris pH 7.5 for 30 min to an hour
1.5 M NaCl
5). Transfer using alkaline transfer as per [1] to nitrocellulose membrane.
6). After transfer, carefully remove gel from the filter and stain with EtBr solution. Photograph the gel for reference
7). UV cross-link filter

Prehybridize 2-4 hours in 9 mL of Jim's hybridization solution without Dextran sulfate with 0.5 mL of Blotto (10% nonfat milk powder in dH_2O) added.
Hybridize with 1-3 x 10^6 cpm of probe per small round filter. Heat probe and 0.5 mL of salmon sperm DNA (10 mg/mL) for 4 min in a boiling water bath, and add to 9.0 mL preheated (67-70°C) Jim's hybridization stock with Dextran sulfate with 0.5 mL of Blotto added.
Jim's Hybridization Stock

15 mL SSC (20x)
10 mL 20% SDS
40 mL Dextran Sulfate
10 ml pyrophosphate
105 mL H_2O
190 mL stock

Hybridize overnight (16 hours) at 65 °C (high stringency) or 55 °C for 72 hours (low stringency).

Low stringency hybridizations were carried out with either entire V or C domain probes at 55°C for 72 hours and then washed at 56°C in 2x SSC and 0.1% SDS 2 x 30 min
High stringency hybridizations were carried out at 65 °C for 16-24 hours and then washed at 65 °C in 0.1xSSC and 0.1% SDS for 30 min.

A15 Colony screening by hybridization

Colony Lifts (As per [1]).

Briefly:

1). Transformed bacteria were placed on 5, 90mm plates and grown 16 hours and then placed at 4°C for 4 hours.
2). Sterile reinforced nitrocellulose filters were labeled, predampened and placed on plates.
3). These lifts were placed colony side up on a fresh plate. The original plate and the new plate with the filter were incubated at 37°C for several hours.
4). A second filter was placed on the first and the "sandwich" was incubated for ~ 2 hours.
5). 4 sets replica filters were produced
6) Filter "sandwiches" were lysed for 2 min on either side by floating them on lysis buffer (0.5M NaOH, 1.5M NaCl).
7) Filters were then treated 3 times for three min with neutralization buffer (0.5M Tris HCl pH 7.5, 1.5M NaCl). In the second treatment the filters are gently separated.
8) Filters were extensively washed with 2X SSC, dried and then UV cross-linked.

A16 *In situ* hybridization (for cryosections)

A16. 1 Synthesis of Hydrolyzed Digoxygenin labeled UTP RNA Probes

1). Linearize 10 µg DNA with appropriate restriction enzyme in 100 µl volume.
2). Add 100 µl 2X Proteinase K Buffer and 5 µl 4 mg/ mL Prot.K.
3). Incubate at 37°C for 30 min.
4). Extract with 200 µl phenol:chloroform, then 200 µl chloroform.
5). Add 25 µl 2.7 M NaOAc + 450 µl EtOH
6). Incubate in dry ice/ethanol bath a minimum of 30 min.
7). Spin at 4°C, 14000 rpm 15 min.
8). Aspirate supernatant, 70% wash, then dry pellet.
9). Resuspend in 40 µl nanopure water.

Transcription Rxn. Mix

4 µl	proteinase K treated DNA (~. 1µg)
5 µl	5X Transcription Buffer (Stratagene)
2.5 µl	10X DIG RNA labeling mix (Boehringer)
1.5 µl	RNasine (Promega)
11 µl	nanopure water
1 µl	appropriate RNA polymerase (Stratagene)
25 µl	

1). Incubate at 37°C for 1 hour
2). Spike the reaction with an additional 1µl of RNA polymerase, then incubate another hour at 37°C
3). Stop the reaction by adding 2 µl 0.5 M EDTA
4). Add 23 µl nanopure to bring volume up to 50µl
(Remove 1 µl aliquot of synthesis mix to run on gel, see below*)
5). Add 50 µl of solution. A
6). Incubate at 60°C for 25 min.
7). Add 50 µl of solution. B
8). Add 1 µl glycogen as a carrier
9). Place reaction mix onto prehydrated centrisep columns
10). Spin 3 min. at 4000 rpm
11). Add 1/10 vol. 3M NaOAc and 2 volumes EtOH (measure volume, there will be loss from the mix passing through the column)
12). Incubate in dry ice/ethanol bath at least 30 min.
13). Spin at 4°C, 14000 rpm 15 min.
14). Aspirate supernatant, 70% wash, then dry pellet.
15). Resuspend in 100 µl depC treated water.
16). Heat at 55°C 10 min. to insure resuspension.
17). Read OD of 10-20 µl final probe.
18). Run 2 µl out on 1.5% agarose/TBE gel at 100 volts to ensure probe was made (one can get a measureable OD without having probe because of the presence of the template DNA)
Hydrolyzed probes should show a smear between 200-500 bp.
*Aliquot of full length probe should be a nice sharp band. If it is not, then there is an RNAse problem.

Pre-hybridization

1). Wash tissues 3X 3 min in PBS
2). Fix tissues in 4% paraformaldehyde/PBS 10 min

Wash 3X 3 min in PBS
3). Digest in Proteinase K (20 ug/mL in PBS) 6 ½ min
(1 mL 4 mg/mL stock/200 mL)
Refix in 4% paraformaldehyde 5 min
Wash 3X 3 min in PBS
4). Wash in 0.1M triethanolamine (TEA) 30 sec.
Acetylate for 10' in 0.25% Acetic Anhydride in TEA
(0.5 mL Acetic Anhydride/200 mL TEA)
Wash 3X 3 min in PBS

Hybridization

1). Make fresh hybridization solution from stocks:
for 1mL.: 500 μl 2X Hybridization solution. (Sigma)
500 μl Formamide
25 μl 10 mg/ml bakers yeast RNA
Final conc.: 5X SSC
1X Denhardt's solution
100 μg/mL DNA
50% formamide
250 μg/mL yeast RNA
2). Dilute probe (1 ng/μl) in hybridization solution and heat at 80°C for 5 min
3). Place 20 μl of DIG probe in hybridization solution onto coverslip
Lay slide down on top of coverslip and allow solution to spread
4). Place slides in a sealed chamber humidified with 5X SSC/50% formamide
Hybridize overnight in incubator at 67°C

Washes and Antibody detection

1). Remove coverslips by dipping slides in 5X SSC preheated to 72°C
2). Wash slides in 0.2X SSC at 72°C for 1 hour
Wash again in 0.2X SSC 5 min at RT
3). Wash slides in Buffer B1 5 min
Block slides in 10% heat-inactivated BSA in B1 1 hour in a sealed chamber humidified with B1
4). Expose to 1/2000 dilution anti-DIG Ab (5μl/10 mL) in B1 with 1% BSA overnight at 4°C in a sealed chamber humidified with B1
5). Wash slides 4X 5 min in B1
Wash in AP Buffer 10 min
6). Develop in the dark in NBT/BCIP diluted in AP until staining reaches desired color

Solutions for *in situ*:

10X Amphibian PBS

NaCl	59g	pH ~7.2
KCl	1.5g	fill to 800 mL
Na_2HPO_4	8.5g	
KH_2PO_4	1.5g	

autoclave
add 0.75g $CaCl_2$
add 0.75g $MgCl_2$-H_2O
fill to 1L

4% Paraformaldehyde

Heat 150 mL dH_2O with 1 drop 10M NaOH on high in microwave for 1min
Add 8 g paraformaldehyde and stir until dissolved
Add 20 mL 10X PBS
Adjust pH to 7.2
Bring volume up to 200 mL with dH_2O

0.1 M Triethanolamine

Add 7.43 g TEA to 350 mL dH_2O
Adjust pH to 8.0 with 10M NaOH
Bring volume up to 400 mL

2X Hybridization solution. (Sigma)

5 mL 20X SSC
200 µl fsDNA (10 mg/mL)
400 µl 50X Denhart's solution
Fill up to 10 mL with DEPC treated H_2O, aliquot

B1

0.1M Tris pH 7.6
0.15M NaCl

For 1 L: 100 mL 1M Tris pH 7.6
30 mL 5M NaCl
870 mL dH_2O

Alkaline Phosphatase Buffer (AP buffer)

100 mM	Tris pH 9.5
50 mM	MgCl
100 mM	NaCl

For 100 mL: 10 mL 1M Tris pH 9.5
5 mL 1M MgCl
2 mL 5M NaCl
83 mL dH_2O

NBT/BCIP (Gibco BRL)

NBT at 100mg/mL in 70% dimethylformamide (DMF) v/v
BCP at 50 mg/mL in dimethylformamide (DMF)

Normal concentration.: 4.5 µl/mL NBT
3.5 µl/mL BCIP

For 30 mL of half concentration: 67.5 µl NBT
52.5 µl BCIP
30 mL AP Buffer

Solutions for DIG probe synthesis

2X Proteinase K Buffer:

0.2 M Tris pH 7.5

25 mM	EDTA
0.3 M	NaCl
2%	SDS

Solutions A and B should be made just prior to use.

Solution A

40 µl	1M $NaHCO_3$
60 µl	1M Na_2CO_3
400 µl	DEPC
500 µl	

Solution B

100 µl	1M NaOAc
5 µl	Acetic Acid
395 µl	DEPC
500 µl	

Gel Solutions and Buffers

50X TAE

12.1 g	Tris
2.86 mL	glacial acetic acid
5.0 mL	0.5 M EDTA pH 8.0

Fill up to 50 mL

10X MOPS Buffer

	41.8 g	MOPS
	4.1 g	NaOAc
	1.86 g	EDTA
ad	450 mL	
Add	16 mL	5M NaOH
Ad	500 mL	

RNA Mini Gel

47.5 mL	DEPC H_2O
5.0 mL	10X MOPS
2.5 mL	formaldehyde
0.6 g	agarose

Add formaldehyde **after** the microwave
Run gel at 80V

References

1. Sambrook, J., E.F. Fritsch, and T. Maniatis, *Molecular Cloning: a laboratory manual.* 2nd ed. 1989, Cold Spring Harbor, NY.: Cold Spring Harbor Laboratory.

Appendix B

Shannon Entropy Alignments

Master Shannon Entropy Alignment

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                                                                                                                        accession#
ecies

uman
EPEVTQTPSHQVTQMGQEVILRCVPI-SNHLY---FYWYRQILGQK-VEFLVS-FYNNEI--SEKSEIFDDQFSVERP-DGSN-FTLKIRSTKLEDSAMYFCAS * 1: L36092
DTAVSQTPKYLVTQMGNDKSIKCEQN-LGHDT---MYWYKQDSKKF-LKIMFS-YNNKEL--IINETVP-NRFSPKSP-DKAH-LNLHINSLELGDSAVYFCAS * 2: U07977
DTEVTQTPKHLVMGMTNKKSLKCEQH-MGHRA---MYWYKQKAKKP-PELMFV-YSYEKL--SINESVP-SRFSPECP-NSSL-LNLHLHALQPEDSALYLCAS * 3: U07977
ETGVTQTPRHLVMGMTNKKSLKCEQH-LGHNA---MYWYKQSAKKP-LELMFV-YNFKEQ--TENNSVP-SRFSPECP-NSSH-LFLHLHTLQPEDSALYLCAS * 4: U07975
ETGVTQTPRHLVMGMTNKKSLKCEQH-LGHNA---MYWYKQSAKKP-LELMFV-YSLEER--VENNSVP-SRFSPECP-NSSH-LFLHLHTLQPEDSALYLCAS * 5: U07978
KAGVTQTPRYLIKTRGQQVTLSCSPI-SGHRS---VSWYQQTPGQG-LQFLFE-YFSETQ--RNKGNFP-GRFSGRQF-SNSR-SEMNVSTLELGDSALYLCAS * 6: L36092
EAGVTQSPTHLIKTRGQQVTLRCSPI-SGHSS---VSWYQQAPGQG-PQFIFE-YANELR--RSEGNFP-NRFSGRQF-HDCC-SEMNVSALELGDSALYLCAR * 7: X61439
ETGVTQSPTHLIKTRGQQVTLRCSSQ-SGHNT---VSWYQQALGQG-PQFIFQ-YYREEE--NGRGNFP-PRFSGLQF-PNYS-SELNVNALELDDSALYLCAS * 8: L36092
DAGVTQSPTHLIKTRGQQVTLRCSPI-SGHKS---VSWYQQVLGQG-PQFIFQ-YYEKEE--RGRGNFP-DRFSARQF-PNYS-SELNVNALLLGDSALYLCAS * 9: L36092
DAGVTQSPTHLIKTRGQQVTLRCSPK-SGHDT---VSWYQQALGQG-PQFIFQ-YYEEEE--RQRGNFP-DRFSGHQF-PNYS-SELNVNALLLGDSALYLCAS *10: L36092
DAGVTQSPTHLIKTRGQHVTLRCSPI-SGHTS---VSSYQQALGQG-PQFIFQ-YYEKEE--RGRGNFP-DQFSGHQF-PNYS-SELNVNALLLGDSALYLCAS *11: L36092
EAGVTQSPTHLIKTRGQQATLRCSPI-SGHTS---VYWYQQALGLG-LQFLLW-YDEGEE--RNRGNFP-PRFSGRQF-PNYS-SELNVNALELEDSALYLCAS *12: L36092
NAGVTQTPKFQVLKTGQSMTLQCAQD-MNHNS---MYWYRQDPGMG-LRLIYY-SASEGT--TDKGEVP-NGYNVSRL-NKRE-FSLRLESAAPSQTSVYFCAS *13: X61446
NAGVTQTPKFRVLKTGQSMTLLCAQD-MNHEY---MYWYRQDPGMG-LRLIHY-SVGEGT--TAKGEVP-DGYNVSRL-KKQN-FLLGLESAAPSQTSVYFCAS *14: X61445
NAGVTQTPKFRVLKTGQSMTLLCAQD-MNHEY---MYWYRQDPGMG-LRLIHY-SVGEGT--TAKGEVP-DGYNVSRL-KKQN-FLLGLESAAPSQTSVYFCAS *15: U07978
IAGITQAPTSQILAAGRRMTLRCTQD-MRHNA---MYWYRQDLGLG-LRLIHY-SNTAGT--TGKGEVP-DGYSVSRA-NTDD-FPLTLASAVPSQTSVYFCAS *16: X61653
NAGVTQTPKFQVLKTGQSMTLQCAQD-MNHEY---MSWYRQDPGMG-LRLIHY-SVGAGI--TDQGEVP-NGYNVSRS-TTED-FPLRLLSAAPSQTSVYFCAS *17: L36092
NAGVTQTPKFRILKIGQSMTLQCTQD-MNHNY---MYWYRQDPGMG-LKLIYY-SVGAGI--TDKGEVP-NGYNVSRS-TTED-FPLRLELAAPSQTSVYFCAS *18: L36092
NAGVTQTPKFHVLKTGQSMTLLCAQD-MNHEY---MYRYRQDPGKG-LRLIYY-SVAAAL--TDKGEVP-NGYNVSRS-NTED-FPLKLESAAPSQTSVYFCAS *19: L36092
NAGVTQTPKFHILKTGQSMTLQCAQD-MNHGY---MSWYRQDPGMG-LRLIYY-SAAAGT--TDK-EVP-NGYNVSRL-NTED-FPLRLVSAAPSQTSVYLCAS *20: L36092
NAGVTQTPKFHILKTGQSMTLQCAQD-MNHGY---LSWYRQDPGMG-LRRIHY-SVAAGI--TDKGEVP-DGYNVSRS-NTED-FPLRLESAAPSQTSVYFCAS *21: X61447
GAGVSQSLRHKVAKKGKDVALRYDPI-SGHNA---LYWYRQSLGQG-LEFPIY-FQGKDA--ADKSGLPRDRFSAQRS-EGSI-STLKFQRTQQGDLAVYLCAS *22: X61444
GAGVSQSPSNKVTEKGKDVELRCDPI-SGHTA---LYWYRQSLGQG-LEFLIY-FQGNSA--PDKSGLPSDRFSAERT-GGSV-STLTIQRTQQEDSAVYLCAS *23: X61442
GAGVSQTPSNKVTEKGKYVELRCDPI-SGHTA---LYWYRQSLGQG-PEFLIY-FQGTGA--ADDSGLPNDRFFAVRP-EGSV-STLKIQRTERGDSAVYLCAS *24: X61440
GAGVSQSPRYKVAKRGRDVALRCDSI-SGHVT---LYWYRQTLGQG-SEVLTY-SQSDAQ--RDKSGRPSGRFSAERP-ERSV-STLKIQRTEQGDSAVYLCAS *25: L36092
GAGVSQSPRYKVTKRGQDVALRCDPI-SGHVS---LYWYRQALGQG-PEFLTY-FNYEAQ--QDKSGLPNDRFSAERP-EGSI-STLTIQRTEQRDSAMYRCAS *26: L36092
GAGVSQSPRYKVTKRGQDVTLRCDPI-SSHAT---LYWYQQALGQG-PEFLTY-FNYEAQ--PDKSGLPSDRFSAERP-EGSI-STLTIQRTEQRDSAMYRCAS *27: L36092
GAGVSQSPRYKVAKRGQDVALRCDPI-SGHVS---LFWYQQALGQG-PEFLTY-FQNEAQ--LDKSGLPSDRFFAERP-EGSV-STLKIQRTQQEDSAVYLCAS *28: M11953
DTGVSQNPRHKITKRGQNVTFRCDPI-SEHNR---LYWYRQTLGQG-PEFLTY-FQNEAQ--LEKSRLLSDRFSAERP-KGSF-STLEIQRTEQGDSAMYLCAS *29: L36092
DSGVTQTPKHLITATGQRVTLRCSPR-SGDLS---VYWYQQSLDQG-LQFLIQ-YYNGEE--RAKGNIL-ERFSAQQF-PDLH-SELNLSSLELGDSALYFCAS *30: L36092
DAEITQSPRHKITETGRQVTLACHQT-WNHNN---MFWYRQDLGHG-LRLIHY-SYGVQD--TNKGEVS-DGYSVSRS-NTED-LPLTLESAASSQTSVYFCAS *31: U17050
DAGITQSPRYKITETGRQVTLMCHQT-WSHSY---MFWYRQDLGHG-LRLIYY-SAAADI--TDKGEVP-DGYVVSRS-KTEN-FPLTLESATRSQTSVYFCAS *32: U17049
DAGITQSPRHKVTETGTPVTLRCHQT-ENHRY---MYWYRQDPGHG-LRLIHY-SYGVKD--TDKGEVS-DGYSVSRS-KTED-FLLTLESATSSQTSVYFCAI *33: U03115
EAEVAQSPRYKITEKSQAVAFWCDPI-SGHAT---LYWYRQILGQG-PELLVQ-FQDESV--VDDSQLPKDRFSAERL-KGVD-STLKIQPAELGDSAMYLCAS *34: M33233
EAGVAQSPRYKIIEKRQSVAFWCNPI-SGHAT---LYWYQQILGQG-PKLLIQ-FQNNGV--VDDSQLPKDRFSAERL-KGVD-STLKIQPAKLEDSAVYLCAS *35: L36092
EAGVVQSPRYKIIEKKQPVAFWCNPI-SGHNT---LYWYLQNLGQG-PELLIR-YENEEA--VDDSQLPKDRFSAERL-KGVD-STLKIQPAELGDSAVYLCAS *36: M33234
DAGVIQSPRHEVTEMGQEVTLRCKPI-SGHNS---LFWYRQTMMRG-LELLIY-FNNNVP--IDDSGMPEDRFSAKMP-NASF-STLKIQPSEPRDSAVYFCAS *37: X07192
DAGVIQSPRHEVTEMGQEVTLRCKPI-SGHDY---LFWYRQTMMRG-LELLIY-FNNNVP--IDDSGMPEDRFSAKMP-NASF-STLKIQPSEPRDSAVYFCAS *38: 02546
DARVTQTPRHKVTEMGQEVTMRCQPI-LGHNT---VFWYRQTMMQG-LELLAY-FRNRAP--LDDSGMPKDRFSAEMP-DATL-ATLKIQPSEPRDSAVYFCAS *39: X07223
AAGVIQSPRHLIKEKRETATLKCYPI-PRHDT---VYWYQQGPGQD-PQFLIS-FYEKMQ--SDKGSIP-DRFSAQQF-SDYH-SELNMSSLELGDSALYFCAS *40: U03115
EAGVTQFPSHSVIEKGQTVTLRCDPI-SGHDN---LYWYRRVMGKE-IKFLLH-FVKESK--QDESGMPNNRFLAERT-GGTY-STLKVQPAELEDSGVYFCAS *41: X06154
DAMVIQNPRYQVTQFGKPVTLSCSQT-LNHNV---MYWYQQKSSQA-PKLLFH-YYDKDF--NNEADTP-DNFQSRRP-NTSF-CFLDIRSPGLGDTAMYLCAT *42: U03115
GEEVAQTPKHLVRGEGQKAKLYCAPI-KGHSY---VFWYQQVLKNE-FKFLIS-FQNENV--FDETGMPKERFSAKCL-PNSP-CSLEIQATKLEDSAVYFCAS *43: L26231
EPGVSQTPRHKVTNMGQEVILRCDPS-SGHMF---VHWYRQNLRQE-MKLLIS-FQYQNI--AVDSGMPKERFTAERP-NGTS-STLKIHPAEPRDSAVYLYSS *44: U03115
NAGVMQNPRHLVRRRGQEARLRCSPM-KGHSH---VYWYRQLPEEG-LKFMVY-LQKENI--IDESGMPKERFSAEFP-KEGP-SILRIQQVVRGDSAAYFCAS *45: L36092
DGGITQSPKYLFRKEGQNVTLSCEQN-LNHDA---MYWYRQDPGQG-LRLIYY-SQIVN---FQKGDIA-EGYSVSRE-KKES-FPLTVTSAQKNPTAFYLCAS *46: U48260
GAVVSQHPSWVICKSGTSVKIECRSL-DFQATT--MFWYRQFPKQS-LMLMAT-SNEGSKA-TYEQGVEKDKFLINHA-SLTL-STLTVTSAHPEDSSFYICSA *47: M11955
HAKVTQTPGHLVKGKGQKTKMDCTPE-KGHTF---VYWYQQNQNKE-FMLLIS-FQNEQV--LQETEMHKKRFSSQCP-KNAP-CSLAILSSEPGDTALYLCAS *48: L36092
DADVTQTPRNRITKTGKRIMLECSQT-KGHDR---MYWYRQDPGLG-LRLIYY-SFDVKD--INKGEIS-DGYSVSRQ-AQAK-FSLSLESAIPNQTALYFCAT *49: M11951
EADIYQTPRYLVIGTGKKITLECSQT-MGHDK---MYWYQQDPGME-LHLIHY-SYGVNS--TEKGDLS-SESTVSRI-RTEH-FPLTLESARPSHTSQYLCAS *50: L27610
EAQVTQNPRYLITVTGKKLTVTCSQN-MNHEY---MSWYRQDPGLG-LRQIYY-SMNVEV--TDKGDVP-EGYKVSRK-EKRN-FPLILESPSPNQTSLYFCAS *51: L36092
DVKVTQSSRYLVKRTGEKVFLECVQD-MDHEN---MFWYRQDPGLG-LRLIYF-SYDVKM--KEKGDIP-EGYSVSRE-KKER-FSLILESASTNQTSMYLCAS *52: U08314
SAVISQKPSRDICQRGTSLTIQCQVD-SQVTM---MFWYRQQPGQS-LTLIAT-ANQGSEA-TYESGFVIDKFPISRP-NLTF-STLTVSNMSPEDSSIYLCSV *53: L36092
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SQTIHQWPATLVQPVGSPLSLECTVE-GTSNPN--LYWYRQAAGRG-LQLLFY-SVGIGQ---ISSEVP-QNLSASRP-QDRQ-FILSSKKLLLSDSGFYLCAW *54: L36092

mouse

VTLLEQNPRWRLVPRGQAVNLRCILK-NSQYPW--MSWYQQDLQKQ-LQWLFT-LRSPGD--KEVKSLPGADYLATRV-TDTE-LRLQVANMSQGRTLYCTCSA.. *1:AE000663
DPKIIQKPKYLVAVTGSEKILICEQY-LGHNA---MYWYRQSAKKP-LEFMFS-YSYQKL--MDNQTAS-SRFQPQSS-KKNH-LDLQITALKPDDSATYFCASSQ *2:AE000663
GPKVLQIPSHQIIDMGQMVTLNCDPV-SNHLY---FYWYKQILGQQ-MEFLVN-FYNGKV--MEKSKLFKDQFSVERP-DGSY-FTLKIQPTALEDSAVYFCASSL *3:AE000663
ETAVFQTPNYHVTQVGNEVSFNCKQT-LGHDT---MYWYKQDSKKL-LKIMFS-YNNKQL--IVNETVP-RRFSPQSS-DKAH-LNLRIKSVEPEDSAVYLCASS. *4:X56725
NTKITQSPRYLIL.GRANKSLECEQH-LGHNA---MYWYKQSAEKP-PELMFL-YNLKQL--IRNETVP-SRFIPECP-DSSK-LLLHISAVDPEDSAVYFCASSQ *5:AE000663
DSGVVQSPRHIIKEKGGRSVLTCIPI-SGHS----NVWYQQTLGKE-LKFLIQ-HYEKVE--RDKGFLP-SRFSVQQF-DDYH-SEMNMSALELEDSAMYFCASSL *6:M15614
NSGVVQSPRYIIKGKGERSILKCIPI-SGHLS---VAWYQQTQGQE-LKFFIQ-HYDKME--RDKGNLP-SRFSVQQF-DDYH-SEMNMSALELEDSAVYFCASSL *7:M15613
EAAVTQSPRNKVTVTGGNVTLSCRQT-NSHNY---MYWYRQDTGHG-LRLIHY-SYGAGN--LRIGDVP-DGYKATRT-TQED-FFLLLELASPSQTSLYFCASSD *8:M15618
EAAVTQSPRNKVAVTGGKVTLSCNQT-NNHNN---MYWYRQDTGHG-LRLIHY-SYGAGS--TEKGDIP-DGYKASRP-SQEN-FSLILELATPSQTSVYFCASGD *9:M15617
EAAVTQSPRSKVAVTGGKVTLSCHQT-NNHDY---MYWYRQDTGHG-LRLIHY-SYVADS--TEKGDIP-DGYKASRP-SQEN-FSLILELASLSQTAVYFCASSD *10:M15616
EAGVTQSPRYAVLQEGQAVSFWCDPI-SGHDT---LYWYQQPRDQG-PQLLVY-FRDEAV--IDNSQLPSDRFSAVRP-KGTN-STLKIQSAKQGDTATYLCASSF *11:AE000664
DAGVTQTPRHEVAEKGQTIILKCEPV-SGHND---LFWYRQTKIQG-LELLSY-FRSKSL--MEDGGAFKDRFKAEML-NSSF-STLKIQPTEPKDSAVYLCASSL *12:AE000664
NAGVIQTPRHKVTGKGQEATLWCEPI-SGHSA---VFWYRQTIVQG-LEFLTY-FRNQAP--IDDSGMPKERFSAQMP-NQSH-STLKIQSTQPQDSAVYLCASSL *13:L29434
DTTVKQNPRYKLARVGKPVNLICSQT-MNHDT---MYWYQKKPNQA-PKLLLF-YYDKIL--NREADTFEK-FQSSRP-NNSF-CSLYIGSAGLEYSAMYLCASSR *14: AE000664
GGIITQTPKFLIGQEGQKLTLKCQQN-FNHDT---MYWYRQDSGKG-LRLIYY-SITEND--LQKGDL-SEGYDASRE-KKSS-FSLTVTSAQKNEMAVFLCASSI *15:AE000664
GALVYQYPRRTICKSGTSMRMECQAV-GFQATS--VAWYRQSPQKT-FELIAL-STVNSA-IKYEQNFTQEKFPISHP-NLSF-SSMTVLNAYLEDRGLYLCGAR. *16: AE000664
DSGVVQNPRHLVKGKEQKAKMDCTPI-NGHSY---VYWYYKKPGEE-LKFLVY-FQNEDI--IDKIDMIGKNISAKCP-AKKP-CTIEIQSSKLTDSAVYFCASSQ *17: X16691
DAAVTQKPRYLIKMKGQEAEMKCIPE-KGHTA---VFWYQQKQSKE-LKFLIY-FQNQQP--LDQIDMVKERFSAVCP-SSSL-CSLGIRTCEAEDSALYLCSSSQ *18:AE000664
VAGVTQTPRYLVKEKGQKAHMSCSPE-KGHTA---FYWYQQNQKQE-LTFLIS-FRNEEI--MEQTDLVKKRFSAKCS-SNSR-CILEILSSEEDDSALYLCASSL *19:M61184
NSKVIQTPRYLVKGQGQKAKMRCIPE-KGHPV---VFWYQQNKNNE-FKFLIN-FQNQEV--LQQIDMTEKRFSAECP-SNSP-CSLEIQSSEAGDSALYLCASSL *20:K02548
DMKVTQMPRYLIKRMGENVLLECGQD-MSHET---MYWYRQDPGLG-LQLIYI-SYDVDS-NSEGDIPKGYR--VSRK-KREH-FSLILDSAKTNQTSVYFCASSL *21:AE000664
SVLLYQKPNRDICQSGTSLKIQCVAD-SQVVS---MFWYQQFQEQS-LMLMAT-ANEGSE-ATYESGFTKDKFPISRP-NLTF-STLTVNNARPGDSSIYFCSSR. *22:X16695
AQTIHQWPVAEIKAVGSPLSLGCTIK-GKSSPN--LYWYWQATGGT-LQQLFY-SITVG---QVESVVQ-LNLSASRP-KDDQ-FILSTEKLLLSHSGFYLCAWS. *23: X03277

laevis

NVVITQEPRSIITRAGSAALMRCEQK-TSDYEW--MFWYQQRDGHG-LQLIST-QLRGDK-PTYEEGYKDG-FQVNRT-ENGKISFLEIKSPMPXDQSLYLCAAS •  1: BV1s1L
NVVITQEPRSIITRAGSAALMRCEQK-TSDYDN--MFWYQQRDGHG-LQLISM-QLRGYD-ATYEEGYKDG-FQVNRT-EKGKISFLEIKSPKPQDQSLYLCAA. •  2: BV1S2L
NVVITQEPRSIITRAGSAALMRCEQK-TSDYDN--MFWYQQRDGHG-LQLISM-QLRGYD-ATYEEGYKDG-FQVNRT-EKEKISFLEIKSPKPQDQSLYLCAA. •  3: BV1S3L
NVVISQEPRSIITRAGSAALMRCEQK-TTDYNT--MFWYQQRDGHG-LQLIST-QLRGDK-PTYEEGYKDG-FQVNRT-TSNKISFLEIKSPKPQDQSLYLCAA. •  4: BV1S4L
GA.VVQIPRSLVVRPGGDVTIECQHN-DSSKLS--KYWYQQEGGKA-LVLMGY-TYTTST-IEMEKEFSEGRFTITPK-STQH-SVLTISGVSAQDSATYYCA.. •  5: BV2L
GIVVTQEKKQLTVNPGDSVALPCHHD-DKSGTYDTMLWYQQKSGGA-LMVLAV-SIGKND-STIEEQFRDT-WTMERP-DIEN-SVLSRGNVAPQDSAMYYCAA. •  6: BV3L
QVTVTQSEKLLLVKRGGFFFFTCHQS-AADYFN--MFWYQQKPGQG-LKLMVW-STGAND-GKMEGEYGQR-WQLHRS-DTHN-CVLNVTGAEAEDDAQYFCAAS •  7: BV4L
GVKVTQVQKLLIIKSGEAAELYCEHD-DSSYYN--MFWYQQKPDQG-LKLMLH-SLNVGS-EDLESDYKDN-WGTDRK-FVLN-STLILKKGNVEDSATYFCAA. •  8: BV5L
GVLVKQEKRLVLVPLGQSADIPCNQD-QSTHFP--MSWYQQNQGEG-LKLMVY-SSGAGD-QNMEKEFGER-WSLNRS-DVYN-SVLTLKEAKMEDAAQYICASS •  9: BV6S1L
GVLVKQEKRLVLVPFGQSADIPCNQD-QSTHLN--MYWYQQNPGEG-LKLMIY-STNEKE-EKMEKEYEER-WSLNRP-DIYN-SVLRLKEAKMEDAAQYFCAS. • 10: BV6S2L
MESVFQEHKLIVVPAGGPVDLYCKHN-HSNSMA--MLWYQQKTGQG-LKLMVY-SSGPNS-GEMEEGFQS--WTLNRP-DIFT-FNLSLKAAGSSDSAEYFCAA. • 11: BV7L
QVKVIQDQKFLAVTLGSSVKVQCSHT-GPSYYA--MLWYQQKLGQG-LKLMVL-SSDTKP-GNMEEGFKE--WSMERP-DTQK-SDLSISQVGAEHSAVYFCAA. • 12: BV8S1L
LINVTQDHRFLAVPERSPVKLHCGHS-ASGFYS--MFWYQQKLGQG-LTLMVL-SSDTKP-GNMEEGFKE--WSMERP-DTQN-SYLCISQVGSEHSAVYFCAA. • 13: BV8S2L
QVKVIQDQKFLAVTLGSSVKVQCSHT-GPSYYA--MLWYQQKLGQG-LKLMVL-SSDTKP-GNMEEGFKE--WSMERP-DTQN-SYLSISQVGAEHSAVYFCAA. • 14: BV8S3L
AAPVLQYPTVQLSHPGDNVTVQCEHN-DGSYTR--KYWYRQENNGP-LTLLG--STYTTQEATVEKDFAGGRIIIQHK-TAQG-AVLIILRVSPQDSALYYCASS • 15: BV9L
GVEVTQKNKLLVVQAGSRVSLECEQN-DKSYFY--MSWYQHRPGQG-LQLMVY-SDDKDS-GNMEKEFTS--WRLKRL-DILN-SNLILESAGSEHSAEYWCAVS • 16: BV10L
GANVTQSPKFLLIKPGESAEIQCHQD-NKNFDY--MYWYQQVPGKE-LKLMML-SRSSAS-VEMESQYEAE-WEAERI-GAMN-SSLRLKKGKVEDSATYFCAA. • 17: BV11L
GLVVTQEKRLLLVLVGGSVEIPCRQD-GDNLY---MYWYQQKAGEG-LELMVV-SAGADAKQTMEGKYAGR-WSLNRT-VIGN-MSLKVTGAEAGDSATYLCSA. • 18: BV12L
SQSIRQSPLVLVTPPGHPVSINCTVD-GADTPN--MYWYRQEPTGGTLLLLSY-SVSKDS---VEQLTLSH-FQPERP-TNSQ-FSIRSSTAQVNDSGTYYCAWS • 19: BV13L
SQTITQTPHILIVPLEGALSLNCSVV-GASDPY--MYWYKQDVGRGGLELIAF-SFGKDS---AEEPTIRH-FQSKRS-SESD-FSLSSKEAKETDGGIYYCAWS • 20: BV14L
G.VVTQEKKLIVAHHGKSLAIPCQHD-ATDYLN--MLWYQQRPGKE-LKLLAI-SAGKGD-TTIENEFKGK-WTMERP-DVLN-STLNRDKVDGEDSAVYYCAS. • 21: BV15L
GVKVIQEPKLVVVQAGKPVKVGCQHN-DSSYIP--MFWYLQQPGLG-LKLMVY-SAGTGL-GDMEEGFKS--WKLERP-EVLE-SKLTLESVGSSNSAVYFCAV. • 22: BV16L
SADVVQTPGLVVSRPGANVSLLCEHK-DSGFLY--KFWYRQEKHRP-LTFIG--SVHTTQRPNMDREFDGRIKIQPQS-AQSA--QLIISNVSPSDAAIYYCTSS • 23: BV17L
AQSITQS.PALTLRPGDKVELRCRVL-GAGDPY--MYWYRQSQGGGTFGFIAS-SVAAGS----EELTLSH-FSAKRT-AKKT-AAANSDGVFLNDSGIYFCAWS • 24: BV19L
DGRVVQVPLLLVARVGAEMSLRCRVT-DGSLSN--TLWYRQGGDGP-LTLIGH-TYRGGT-PSIESSFSGTNITIRAE-STDS-SLLLISGVSGQHSGTYFCAGS • 25: BV18L

gilli

NVVITQEPRSIITTAGSAALMRCEQK-TSDYEW--MFWYQQRDGHG-LHPIST-QLRGDK-PTYEEGYKDG-FQVNRT-TSAKISFLEIKSPKPQDQALYLCAA  •  1: BV1s1G
NVVITQEPRSIIIRAGSAALIRCEQK-TSDYDN--MFWYQQRDGHG-LQLISM-QLRGYD-ATYEEGYKDG-FQVNRT-EKEKISFLEIKSPKPQDQSVYLCAA  •  2: BV1S2G
NVVIAQEPRSIIVRAGSAALMRCEQK-TTNYNT--MFWYQQRDGHG-LQLIST-QLRGDK-PTYEEGYKDG-FQVNRT-TGDKISFLEIKSPKPQDQALYLCAA  •  3: BV1S3G
..................VTIECQHN-DSSKLS--KYWYQQEGGKA-LVLMGY-TYTTST-VEMEKEFSEGGFTITPK-STQH-SVLSISGVSAQDSPTYYCAS  •  4: BV2G
QVTVTQSEKLLLVERGGSVRIPC.QS-AADYFN--MFWYQQKPGQG-LKLMVW-STGAND-GKMEGEYGQR-WQLHRS-DNHN-SVLNVTGAEAEDDAQYFCAA  •  5: BV4G
GVKVTQVQKLLMIKSGEGAELYCEHN-DNSYYN--MFWYQQKPDQG-LKLMLH-SLNVGS-EDLESDYKAN-WGTERK-FVLN-STLRLKEGNVEDSATYFCAA  •  6: BV5G
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          GVLVKQEKRLVFVPLGQSADIPCNQD-QSTHLN--MYWYQQNSGEG-LKLMIY-STNEKE-EKMEKEYEER-WSLNRP-DIYN-SVLRLKEAKMEDAAQYFCAS  *   7: BV6S1G
          QGVLVKQKRLVLVPLGQSADIPCNQD-QSTHFA--MTWYQQNQGEG-LKLMVY-SSGAGD-QNMEKEFGER-WSLNRS-DVYN-SVLMLKEAKMEDAAQYICAS  *   8: BV6S2G
          MESVFQEPRLIVAPAGGPVDLYCKHN-HSNSMA--MLWYQQKTGQG-LKLMVY-SSGPNS-GEMEEGFQS--WTLNRP-DITF--NLSLKAAGSSDSAEYFCAA  *   9: BV7G
          QVKVIQDQKFLAVTLGSSVKVQCSHT-GPSYYS--MLWYQQKLGQG-LKLMVL-SSDTKP-GNMEEGFRE--WSMQRP-DTQN-SDLIISQVGAEHSAVYFCAA  *  10: BV8S1G
          LINVTQDHRFLVVPERSPMKLHCSHS-ASGFYS--MLWYQQKLGQG-LTLMVL-SSDTKP-GNMEEGFKE--WSLERP-DTQN-SYLSISQVGAEHSAVYFCAA  *  11: BV8S2G
          Q.KVIQDQKFLAVTLGSSVKVQCSHT-GPSYYA--MLWYQQKLGQG-LKLMVL-SSDTKP-GNMEEGFKE--WSMERP-DTQN-SYLSISQVGAEHSAVYFCAA  *  12: BV8S3G
          AAPVVQYPTVQLSHPGDNVTVQCEHN-DGSYTR--KYWYRQENNGP-LTLLG--STYTTQ-EATVEKDFVGGRIIIQH-KTAQGAVLIILRVSPQDSALYYCAS  *  13: BV9G
          GANVTQSPKFLLIKPGKSAEIHCHQD-NKNFDY--MYWYQQVPGKE-LKLMML-SRSSAT-VEMETQYEAE-WEAERI-GAMN-SSLRLKKGKVEDSATYFCAA  *  14: BV11G
          GLVVTQQKRLLLVAVGGSVEIPCRQD-GDNLY---MYWYQQKAGEG-LELMVI-SAGANA-KQTMEGKYTGRWSLNRT-VIGN-MNLKVTGAEAGDSAIYLCSV  *  15: BV12G
          .QSIRQSPLVLVTPPGHPVSINCTVD-GAGTPN--TYWYRQEPTGGTLLLLSY-SVSKDS-VEQLTLSH---FQPERP-TNSQ-FSIRSSTAQVNDSGTYYCAW  *  16: BV13G
          .QTITQTPHILIVPLEGAVSLNCSVV-GASDPY--MYWYKQDVGRGGLDLIAF-SFGKVS-TEVLTIRH---FQVKRS-SDSD-FSLSSKEAKETDGGIYYCAW  *  17: BV14G
          GVVVTQEKKLIVAHHGKSLAIPCHHD-ATDYLN--MLWYQQRPGKE-LKLLAI-SAGKGD-TTIENEFKGK-WTMERP-DVLN-STLNRDKVDGEDSAVYYCAS  *  18: BV15G
          GVKVIQDPKLVIVQAGKPVNVGCKHN-DSSYIP--MSWYLQQPGQG-LKLMVY-SAGTGL-GD-MEEGFKS-WKLERP-EVLE-SKLTLESVGSSNSAVYFCAV  *  19: BV16G
          SADVAQTPGLVLSRPGANVTLLCEHK-DSGFLY--KFWYRQEKHRP-LTFIG--SVHTTQ-KPNMDREFDG-RINIQP-QSAQSAELTISNVSPSDAAMYYCTS  *  20: BV17G
          .QSITQS.PALTLRPGDKVELSCRVP-GAGDPY--MYWYRQSQGRGTFGFIAS-SVAAGS-VEELTLSH---FSAKRT-SKEN-FSLSSDGVFLNDSGIYFCAW  *  22: BV19G
axolotl

          PQTIHQNPRFPSPAAGEPVEMTCSVK-GYDNGPV-MYWYRKFRSEP-FEMLFL-SRTTGM---LDDSAEKH-FKAERP-GAQD-FILRTESLLPNDTAVYFCRW * 1: axoBV7.1
          SDAAVVQTPWVVRRTGDSAQLPCEQS-DNHNY---MYWYQQPPGGG-LQLIWY-SLGENV--VDQENKTWARFSARRA-STRI-FTLEIKELEPRDSAVYFCAS * 2: axoBV1.1
          SQRIIQRPRSLVQSAGSPALLECSVE-GASTPY--MYWYRQLHTQT-LTLMSY SVTTKS---VEENNIQH-FTGVRP-TGDT-FILKTERLEPNDTALYYCAW * 3: axoVB6
          SASYVEQPAFLFGDPGSSTAISCRLK-DSAKPW--MYWWRKRPGEL-EG.LFY-ALMAEVV----SNFTAEQLAAERR-DMH--FELTFPQLRPSNSALYYCA. * 4: axoBV8
          QAIISQSPQSIAEWKGGPASLYCSVK-GTSNPN--LYWYRQTPSSG-LQMLSY-SISAGS---IAENVLRH-FTTKRP-EDLA-FTLETTRLELSDTAVYYCA. * 5: axobv5
          SNAALLQTPYTVKRPGESADVQCVQD-TGHNA---MYWYQQVPGGG-LKPLYY-SLAPND-ATREENVQLS-FSASRP-ETKS-FTIDITGFEPDDSAVYFCA. * 6: axobv2
          PQTIHQSPRFASPAAGEPLEMTCIVK-GYDNPH--MYWYRKFPDKP-FEMLFF-SRATGM---LDDTAEKH-FKAERP-GAQD-FILRTESLLPTDTAVYFCAR * 7: axoVB7.2
          GVCAGVQVPEGRSITSGPVKMTCEHT-DPSFIT--MLWYRQSNGSL-TLIVTS-VSGSDP--TFEGSFQSG-YELERP-QVLQ-ASLKILAGARGDSATYFCAR * 8: axoBV4
          SEAAVWQKPHVMERAGISAQLLCEQD-DNHVA---MYWYQQPPGRE-LQLICY-SVGENN--VDIEKMTWARFSARRP-STRI-FTLVIKEMEHRDSAVYFCAS * 9: axoBV1.2
          TPSYVEQPPFVTGLSGGKAEITCKLI-DSSKPQ--MYWYRKHRERG-FEALFS-AYSAGE----AVNYTAEGMTAERI-GDN--FPLQLPKLQSSDSGLYYCVR * 10: axoBV9
          AGQFVVQPKYTVATEGERVTVQCHLQ-DSAYYW--MYWYRQESRGGELQALV--SSGGKD-DLSYLTNGNNTFEAERP-KKED-FRLVRASAMPHHASVYFCAC * 11: axoBV3
shark

          SALLTQWGNRLMVAEGTTAEMHCFQN-DTNETY--MYWYRQHSGAW-LQLIVN-SIGTSD--PRPEEGFKERFKVTRP-DFKS--CSLKIMRVDQTDRAVYYCAA * 1: L47431
          SALITQWGDRLTVAEGKTAEMHCFQN-DTDYTY--MYWYRQHSGAG-LQLIVT-SVGTSD--STPEDGFKERFKATRP-DLKT--CSLKILRVDQTDRAVYYCAA * 2: L47435
          SALVTQWGNRLTVAEGKTVEMHCFQN-DTSDSY--MYWYRQQSGAG-LLLIVT-SIGTSD--TSPEEGFKERFKVTRP-DLKT--CSLKILRVDQTDRAVYYCAA * 3: L47440
          AVLIQQTPASISHSPGSPVRIQCIYT-EATAGSY-FYWYRWHLDR-EPQDLFY-SSYAGT--VNPSGEVDG-FTARRP-ENTH--FYLESSSLWVNQSAVYYCAW * 4: L47432
          APTVVQTPAAASIRPGKPLALGCSLE-GASMGSYTMHWYRQEGKAVEWMCNEH-GSCGPG--FEKRFTVQHDSASNR--------FSLQLGDPQPTDTATYYCAA * 5: L47433
          AEVIHQWPTLVAVKGGEPAELNCYQN-DSSKSV--MLWYRQYAGQG-LTLMGH-SYTGRA--PIYEGKFEEEVKIIRP-EERR--CSLRVLKGKAVDAAVYYCAA * 6: L47434
          AEVIHQWPTLVAVKRGEAAELNCYQN-DSSKSV--MLWYRQYAGQG-LTLMGY-SYTGSS--PTYEGKFEEEVKIIRP-EDKR--CSLRVLKVKAVDAAVYYCAA * 7: L47438
          AEVIHQWPTLVAVKGGEPAELNCYQN-DSRKSI--MLWYRQYAGQG-LTLMGY-SFTGSS--PTYEGKFEEEVKIIRP-EDKR--CSLRVLKVKAADAAVYYCAA * 8: L47455
          AEVIHQWPTLVAVKGGEPAELNCYQN-DSSKSV--MLWYRQYAGQG-LTLMVI-LLMGIP--VTYEGKFEEEVKIIRP-EDKR--CSLRVLKVKAADAAVYYCAA * 9: L47449
          RVLQAPTAVSVSRFPGEGVGLECRVE-GESSGS--MFWYRQDPGK-EPRLMFY-SAATPS--VNSEGTVEG-FTAKRP-TGSH--FNLESTSLRENHSALYFCAW *10: L47437
          SKNIHQTPAAVSRFPGKGMELRCTVE-GSSSTLY-MYWYRQEPGK-EPLLMFY-SNTANY--VDPEGTVDG-FTAERP-TDSQ--FNLESSSL.ENHSAVYFCAW *11: L47451
          SQTIHQTPASVSKFSEEQVKLKCTVD-GTSNPY--MYWYRQYPGK-EPQMMFY-SLTAQR--ENSEGTVEG-FTAERP-TDSE--FILESSSLRENHSAVYFCAW *12: L47441
          SSYVEQSPSILSIAAGESTTLRCTLT-NTDYSF--LYWYRQQPDRA-LEALFY-SATEGS--VTN-YTSES-FKADRA-SNTL--FTLALRDAAPSHTAVYFCTC *13: L47442
          ASYVEQSPSILSISAESRTTLRCTLT-NTAYSY--MYWYRQQPDRA-LEALFY-SVGTGS--VTN-YTSES-FKADRA-SNTL--FTLALRDAAPSHTAVYFCTC *14: L47448
          SSYVEQIALYPIDFRRESTTLRCTLK-NTGYPA--MYWYRQQPDRA-LEALFY-SVGTGS--VTN-YTSES-FSADRA-SNTL--FTLALRDAAPSHTAVYFCAC *15: L47444
          GDVIHQWPQSLAVKQPGPVEMNCLQK-GKIRDY--MYWYRQSPGGGFLSMGSV-YLGNE---PQYEEGFKSGFVITRTLDNKK--STLTIESVNATDEAVYFCAA *16: L47443
.rout
          SNQVHQTPADLYKNQGKSAKMECSHS-ISGYNR--ILWYKQSNYRE-LVLLGY-MIGTSG---YPEAGFDIEGDANAGGT-----STLTIKQLTPNSSAVYYCAD * 1: U18124
          GSGVTQTPTILWELKESDALMNCSHT-KDSSYYQ-MYWYRQLPGEGM-KQVVF-IIPSSQ--PDYSGEFSKDKYPTVKAVAES--GSFTVKKLETGDSGMYFCAV * 2: U18123
          TDKVHQTPTAILTGPEDKVNLTCSHT-DPNYNM--ILWYQQSAQNTALKLIGY-VWNTS---PTVEDSFKGRFDVSGDAAANKMAYLHFPKVTEAEDSAVYFCAA * 3: U18125
          SKSVYQSPSAAL.KPN.SVTLNCSHK-IPRYDT--VLWYKRSVEDTALNLIAY-VYKPQQ--LNPHIKVTLTCKGDGESNA----FLHILKPRQAEDSGEYFCAG * 4: VB4
atfish
          AKDVHQTPPDLIKNIQESTDLSCSHS-IPNHEF--MLWYKRSENKQ-LQLLGY-LNSKFP---YPEDSLKAKIELYGD GNNE--GKLTIKNLQPDDSAVYFCAV * 1:AF178029
          LNIQQTPQRLLMTTEQTEAKLKCHHG-DASYPY--MYWYQQKTVSDSIELIGM-LQYGTS---TPEEKFKARFNISGH-ATGDA-FLL-ISSITTEDSAVYFCAA * 2:AF178028
          CKDVHQNPTDVLCLPEESVTLTCKHS-IPNYIT--ILWYQRTHGDTHLKLIAY-VFYTTP---KYEGNYEGNFTVSGD-GQSSA-SLQISKATQVLHSALYFCAA * 3:AF178027
          TCAKVQQTPSILVNEKENITVSCSHN-DNNLDR--MLWYRQNSRTV-LALIGY-TMTAKSD-PKYEEEFNDRFTLSRQ-STLA--GTLTISNLRQSDSAVYYCAA * 4:AF178026
          GVLITQWPKYISGFKSTSVDMHCYQN-DTDYDY--TYWYRQIEGKE-PVLIAR-YVAGS---PIQEKGFENGFKAWGT-KKK---WSLTVDVEEDSD-AVYLCAA * 5:AF178025
          ....................................YWYQQNSRTV-MALIGY-TAGASSD-PNYEGGFKDRFKQSRQ-GTLN--GSLTISNLRQSDSAVYYCAA * 6:AF178024
          .NCVKFEQTPSILATETSEITIQCSH-DDSSLYT-MLWYQQNSHTV-MALIGY-TAGASGG-PNYEDGFKDRFKQSRQ-GTLK--GNLTISSLRQSDSAVYYCAA * 7:AF178023
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      .LIQVQQEPDDLVLTPGSSVKVSCAI-TGTNNPD-LFWYRWNEAAG-FVLVFS-SRGAGMMDPVSEGQFK----SSRP-TDLQ--MVLESEGLSEIGSAVWYCAA * 8:AF178022
      .SDLVFQTPPDLFGNHKQSVKIQCVH-SVPSYNQ-INWYRETQDQG-LTLIGY-QYRTSS--PQIENDFKLKVEIAGD-GNKN--VSLTIKNLSSNDSVVSFCAA * 9:AF178021
raja
       --EIQQSPNSLSVSEGGQGRMSCLQKGTVRDS--KLWYRQTEGAG-LELIG--SVYSSQ--ESQYEKEFRSGYFISADAGNK--FSLEIRSVRDRDEARYFCAA * 1: U75756
       --TVPQTPDTLSISPDQKMEVKCILEGGSSSSY-MYWYRQYPGAGPQIMFS--SVGEGN--VQ-PDNTMAGLKAERP-NLSE--FYLKSSGSVANSTAVYFCAW * 2: U75753
       --SVHQSPGALTRSPGQTVKVKCIQQDSSGY---IYWYRQYSGAGAQNLFY--SAAANI--VV-PPPPVTGFTAERP-NNNE--FYLKSSGLEADSSAVYFCAW * 3: U75769
       --TVHQSPGALSGSPGEKVEVECTIEGSSNPADRLFWYRQYRGEGLHNLFY--SFGVND--VQ-SSKSPAGFTAERP-RSHV--FFLKSTDLKADLPAEYFCAW * 4: U75752
       --TIHQTPDALSASPGQKIQVKCTIEGGSGIEN-MYWYRQYPGVEPENLFY--SVGIKN--VQ-PDEQVSGFTANRP-NNNE--FYLKSASLAAKSSAQYFCAW * 5: U75754
       --TVRQIPTTVSGETGNILELRCTAEGGGNTDY-MYWYGQPAGEGLRNLFH--STYQGH--VE-SKETANGLSAVR--KDNR--FDLKFTGLAANHSAQYFCAW * 6: U75751
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Appendix C

Repertoire Data

cDNA libraries as indicated in the first column were screened with 10 Vβ probes indicated along the top of the figure. Two libraries were screened per condition. Five plates of colonies were screened in each case (e.g., A1-A5 and N1-N5).

Hybridizing colonies were counted twice and tabulated.

In data sets with one missing value, the missing value was replaced with the sample mean of the 10 plate counts for that condition and indicated as read. BH represents blots that could not be reliably read. ND stands for not done. Where there were multiple missing values in for Vβ segment counts, the remainder of the segments counts for that condition were scaled to match the least complete data (i.e. where only 7 of 10 plate counts were available for a particular Vβ segment for a particular RACE library, the data for all other Vβ plate counts from that same library were scaled by multiplying by 0.7). This leads to an underestimation of the actual significance of the data because it reduces the number of colonies scored to allow comparison with smaller samples. It does however allow more samples to be compared. The samples were normalized by calculating the proportion of the total number of Vβ positive colonies. These values were used to generate the figure 5.5.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	sums
A1		11	0	25	6	0	16	ND	8	0	0	7	0	1	0	ND	0	2	1	77
A2		5	2	40	4	1	34	ND	15	2	6	18	0	2	12	ND	1	3	12	157
A3		21	0	35	2	0	31	ND	17	6	1	13	0	8	5	ND	0	2	7	148
A4		25	2	38	5	8	29	ND	23	1	0	11	0	7	8	ND	5	2	16	180
A5		11	1	29	3	5	25	ND	20	4	1	0	0	1	9	ND	0	2	9	120
N1		3	0	7	ND	0	BH	1	0	0	ND	0	3	1	0	24	0	1	2	42
N2		4	0	2	ND	0	BH	3	0	0	ND	0	0	0	2	9	0	0	2	22
N3		4	0	10	ND	1	BH	4	0	0	ND	2	1	2	0	4	0	0	2	30
N4		0	0	1	ND	1	BH	3	0	0	ND	0	0	1	0	4	0	0	2	12
N5		1	0	6	ND	1	BH	5	0	0	ND	1	0	1	0	10	0	1	2	28
	TOTAL	42.5	2.5	96.5	20	8.5	135	16	41.5	6.5	8	26	2	12	18	51	3	6.5	27.5	523
LG3 tadpole spleen	% of total repertoire	8.13	0.48	18.5	3.82	1.63	25.8	3.06	7.93	1.24	1.53	4.97	0.38	2.29	3.44	9.75	0.57	1.24	5.26	100
B1		13	0	7	3	3	29	ND	10	3	0	3	0	1	1	ND	0	0	2	75
B2		25	5	5	15	1	19	ND	7	5	0	9	0	0	7	ND	0	4	3	105
B3		27	0	6	6	2	13	ND	10	1	1	3	2	3	1	ND	4	0	1	80
B4		29	2	16	2	3	45	ND	9	4	1	6	0	10	5	ND	5	2	2	141
B5		32	3	8	6	2	32	ND	9	3	1	8	0	3	4	ND	1	1	2	115
M1		9	0	7	ND	0	BH	1	0	0	ND	1	0	0	0	3	0	1	2	24
M2		10	0	2	ND	0	BH	3	0	0	ND	0	0	1	0	3	0	0	2	21
M3		11	0	10	ND	1	BH	4	0	0	ND	0	0	1	2	2	0	1	2	34
M4		11	0	1	ND	1	BH	3	0	0	ND	0	0	1	1	7	0	0	2	27
M5		11	0	6	ND	1	BH	5	0	0	ND	0	3	2	1	2	0	0	2	33
TOTAL		89	5	34	32	7	138	16	22.5	8	3	15	2.5	11	11	17	5	4.5	10	431
LG3 tadpole thymus	% of total repertoire	20.7	1.16	7.9	7.43	1.63	32.1	3.72	5.23	1.86	0.7	3.48	0.58	2.56	2.56	3.95	1.16	1.05	2.32	100
C1		22	2	4	2	2	28	ND	12	9	0	7	0	1	6	ND	3	4	6	113
C2		14	6	18	3	2	46	ND	6	5	0	2	0	3	1	ND	1	4	2	113
C3		22	0	6	5	0	10	ND	7	11	0	0	0	5	2	ND	0	1	2	71
C4		12	1	12	7	2	40	ND	8	9	1	5	0	2	3	ND	5	6	3	116
C5		39	2	11	3	0	16	ND	0	0	0	2	0	5	1	ND	1	3	0	83
G1		29	2	0	5	5	7	7	28	22	0	2	1	8	4	12	4	9	1	146
G2		26	1	6	6	1	6	6	19	21	2	2	2	6	9	7	4	6	0	130
G3		22	0	4	2	6	8	8	28	11	0	2	1	5	3	21	4	4	6	133
G4		29	0	7	9	15	8	8	18	15	3	2	0	5	14	12	10	4	2	161
G5		24	2	18	6	6	12	12	14	13	0	1	1	2	6	8	3	5	1	134
TOTAL		120	8	43	24	19.5	90.5	41	69	58	3	12.5	2.5	21	24.5	60	17.5	23	11.5	648
LG15 tadpole spleen	% of total repertoire	18.4	1.23	6.64	3.7	3.01	14	6.33	10.6	8.95	0.46	1.93	0.39	3.24	3.78	9.26	2.7	3.55	1.77	100
D1		16	2	8	18	4	34	ND	12	21	0	6	0	7	16	ND	0	5	8	157
D2		9	0	17	10	10	25	ND	13	15	0	4	1	3	14	ND	6	9	8	144
D3		12	5	11	19	10	35	ND	13	14	0	3	4	4	18	ND	4	10	10	172
D4		5	1	17	25	7	37	ND	18	12	1	10	2	7	9	ND	3	7	10	171
D5		3	2	8	41	3	32	ND	6	14	0	1	0	6	4	ND	5	6	0	131
TOTAL		45	10	61	113	34	163		62	76	1	24	7	27	61		18	37	36	775
LG15 tadpole thymus	% of total repertoire	5.81	1.29	7.87	14.6	4.39	21		8	9.81	0.13	3.1	0.9	3.48	7.87		2.32	4.77	4.65	100
E1		4	2	7	7	6	22	12	10	8	1	0	0	3	4	11	2	1	0	77
E2		8	2	11	3	6	25	5	15	5	0	4	2	0	1	13	4	1	1	106
E3		7	1	13	3	6	20	7	15	5	2	4	3	2	2	15	5	2	1	113
E4		16	2	17	1	14	33	20	17	10	1	8	2	1	4	15	1	0	3	165
E5		8	2	7	0	13	29	5	8	8	1	1	0	1	0	10	2	0	0	95
H1		32	1	0	4	9	1	1	4	0	0	BH	0	7	1	9	4	0	1	74
H2		30	0	0	1	12	2	2	4	0	0	BH	0	6	2	9	4	1	2	75
H3		17	0	5	1	7	3	3	12	3	0	BH	0	4	4	8	3	1	1	72
H4		8	4	0	2	13	1	1	2	1	0	9	0	5	0	5	2	1	0	54
H5		17	6	3	3	2	3	3	15	2	0	4	0	4	0	5	6	0	1	74
TOTAL		103	14	44.1	17.5	61.6	97.3	41.3	71.4	29.4	3.5	30	4.9	23.1	12.6	70	23.1	4.9	7	659
LG15 adult spleen	% of total repertoire	15.6	2.13	6.7	2.66	9.35	14.8	6.27	10.8	4.46	0.53	4.56	0.74	3.51	1.91	10.6	3.51	0.74	1.06	100
F1		22	0	3	4	20	11	11	6	3	2	1	0	0	1	14	5	2	0	105
F2		31	3	14	2	19	12	12	13	3	1	0	0	2	6	18	2	1	3	142
F3		42	2	16	5	19	7	7	10	7	1	9	1	3	4	20	0	3	2	158
F4		28	1	11	4	25	10	10	13	3	2	0	1	2	3	18	5	0	1	137
F5		31	0	9	4	10	11	11	9	6	0	1	1	0	3	20	1	2	2	121
I1		47	0	19	8	6	BH	12	12	10	0	8	0	0	2	26	1	5	0	156
I2		48	0	0	13	7	BH	19	10	1	3	15	0	0	5	30	7	4	0	162
I3		61	0	2	20	21	14	12	24	9	0	23	1	1	5	41	6	1	0	241
I4		63	0	22	14	16	9	20	29	6	4	21	1	0	22	38	10	2	0	277
I5		42	0	2	10	11	15	8	18	6	2	15	0	6	17	30	7	2	3	194
TOTAL		332	4.8	78.4	67.2	123	89	97.6	115	43.2	12	74.4	4	11.2	54.4	204	35.2	17.6	8.8	1372
LG15 adult thymus	% of total repertoire	24.2	0.35	5.71	4.9	8.98	6.49	7.11	8.4	3.15	0.87	5.42	0.29	0.82	3.96	14.9	2.57	1.28	0.64	100
J1		77	0	2	11	16	rem	14	14	9	10	16	3	2	8	37	10	4	0	233
J2		82	0	4	24	13	rem	6	22	9	7	15	1	2	9	38	8	2	0	242
J3		90	1	8	10	10	rem	4	23	13	11	17	0	5	11	40	10	2	0	255
J4		53	0	6	15	1	BH	15	6	14	0	0	0	5	9	35	2	0	0	161
J5		51	0	31	20	0	BH	13	4	16	1	5	0	4	11	38	8	3	0	205
K1		36	0	5	ND	4	BH	5	0	0	0	6	0	4	0	14	1	0	3	78
K2		37	0	6	ND	5	BH	2	0	0	0	4	1	5	0	6	0	0	2	68
K3		21	0	8	ND	13	BH	1	0	0	0	0	1	5	0	6	0	0	0	55
K4		38	0	5	ND	10	BH	10	2	0	0	0	1	8	0	10	1	0	2	87
K5		31	0	7	ND	4	BH	2	1	0	0	10	0	1	1	6	1	0	2	66
TOTAL		258	0.5	41	80	38	0	36	36	30.5	14.5	36.5	3.5	20.5	24.5	115	20.5	5.5	4.5	765
LG3 adult thymus	% of total repertoire	33.7	0.07	5.36	10.5	4.97		4.71	4.71	3.99	1.9	4.77	0.46	2.68	3.2	15	2.68	0.72	0.59	100
L1		4	0	4	ND	3	BH	2	1	0	0	0	0	4	1	4	0	5	0	28
L2		7	0	7	ND	1	BH	3	0	0	0	0	0	4	1	19	2	0	1	45
L3		9	0	3	ND	7	BH	7	0	0	0	0	0	8	0	5	2	0	3	44
L4		6	0	10	ND	12	BH	0	1	0	0	2	0	14	2	8	0	0	0	55
L5		7	0	3	ND	11	BH	2	0	0	0	0	0	10	1	7	0	0	1	42
O1		4	4	20	ND	51	BH	8	5	0	ND	4	2	0	7	21	7	ND	0	133
O2		2	2	25	ND	50	BH	10	6	3	ND	8	1	2	6	31	6	ND	0	152
O3		2	2	26	ND	30	BH	7	7	6	ND	3	1	1	4	30	4	ND	0	123
O4		4	4	31	ND	31	BH	BH	1	2	ND	3	0	0	0	24	2	ND	4	106
O5		13	0	10	ND	26	BH	BH	3	1	ND	5	0	0	2	39	6	ND	13	118
TOTAL		58	12	139		222		39	24	12	0	25	4	43	24	188	29	5	22	846
LG3 adult spleen	% of total repertoire	6.86	1.42	16.4		26.2		4.61	2.84	1.42	0	2.96	0.47	5.08	2.84	22.2	3.43	0.59	2.6	100